DETECTION OF TYPE-SPECIFIC ANTIBODY TO HEPATITIS C VIRUS AND ITS APPLICATION FOR SEROLOGICAL TYPING

by

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A thesis submitted in part fulfillment of the requirements of the University of Edinburgh for the Degree of Doctor of Philosophy.

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To my parents and grandparents

DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.

CONTENTS

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ABBREVIATIONS

ALT	Alanine aminotransferase
ATP	Adenosine triphosphate
b.p.	base pairs
BVDV	bovine viral diarrhoea virus
BSA	bovine serum albumen
BW	bead wash
CMV	cytomegalovirus
CAH	chronic active hepatitis
cDNA	complementary DNA
CPH	chronic persistent hepatitis
CTL	cytotoxic T lymphocytes
°C	degrees Celsius
DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EBV	Epstein Barr virus
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HChV	hog cholera virus
HIV	human immunodeficiency virus
HVR	hypervariable region
IRES	internal ribosome entry site
Ig	immunoglobulin
IPTG	isopropyl-thiogalactosidase

IVDA	intravenous drug abuse
kDa	kilodalton
LiPA	line probe assay
M	molarity
MCAC	metal chelate affinity chromatography
MHC	major histocompatability complex
MOI	multiplicity of infection
μg	microgram
μl	microlitre
mg	milligram
ml	millilitre
mM	millimolar
NANBH	non-A non-B hepatitis
ng	nanogram
nm	nanometre
NCR	non coding region
NR	non reactive
NTS	non-type-specific
OAS	2'5'-oligo-adenylate synthetase
O.D.	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RFLP	restriction fragment length polymorphism
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid

rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Taq	*Thermus aquaticus*
u.v.	ultra violet
V	Volts

ABSTRACT

Hepatitis C virus (HCV) is the major causative agent of non-A non-B hepatitis worldwide, often leading to chronic disease, cirrhosis and hepatocellular carcinoma. HCV displays high levels of genetic heterogeneity, and is currently classified into six major genotypes, which can be further divided into subtypes. Genotypes of HCV show distinct geographical distributions with type 1, 2 and 3 infections occurring in a wide range of areas whilst types 4, 5 and 6 are generally confined to more specific regions. The clinical relevance of different genotypes is becoming increasingly evident, with type 1 infections being associated with a low rate of sustained response to interferon therapy, and arguably a more rapid course of disease. Many genotyping assays have been described which involve the analysis of subgenomic regions of RNA, such as the 5' non coding region (5'NCR), that have been amplified by the polymerase chain reaction (PCR). Serological determination of HCV genotype is also possible as high levels of amino acid variation within certain immunodominant regions of the genome often generate a genotype-specific antibody response. The foundation of this project was the development of an indirect serotyping ELISA for the identification of genotypes 1-3. This thesis is concerned with the further development of the serotyping ELISA.

Initially, the assay was extended by the incorporation of antigen for recently discovered genotypes 4, 5 and 6. This version of the assay was able to detect type-specific antibody in 87% sera from blood donors and patients with chronic HCV

infection from various geographical areas. The specificity of this assay was >97% when compared to genotyping by a PCR method. This serotyping ELISA was applied to a number of different population studies, including the U.S.A., Norway, Pakistan, Egypt and Hong Kong, and in doing so has contributed to current knowledge of genotype distributions worldwide.

The availability of a large set of samples from blood donors in Hong Kong lead to an extensive study of HCV type 6, which has a restricted geographical distribution (Hong Kong, Macau and Vietnam) and for which analysis had previously been limited. Type 6a accounted for 27% infections of blood donors in Hong Kong, although type-specific antibody was detected in only 75% these infections by the serotyping assay. Sequence analysis of type 6a core and NS4 regions revealed that the reduced sensitivity of the ELISA to this genotype was not due to antigenic variation, and that high levels of sequence similarity exist within this subtype. Analysis of the risk factors for these donors revealed that a significantly greater number of those infected with type 6a had reported a history of drug abuse (66%) than donors infected with type 1b (7%).

In an attempt to improve the sensitivity of the assay, peptides corresponding to an additional immunogenic region in NS4 were included and assessed for their contribution to the sensitivity and specificity of the assay. The performance of the serotyping assay was also compared to other PCR-based methods of genotyping. An investigation into samples producing discrepant results in two distinct cohorts was performed by sequence analysis of the 5' NCR, core and NS4 regions. The

frequency of discrepant results was higher when testing samples from haemophiliacs than from patients with chronic HCV who had no history of multiple exposure to the virus. Sequence analysis revealed that mis-typing by the ELISA may have resulted from amino acid changes within NS4 from the non-haemophiliac study group, whereas little antigenic variation was identified in discrepant haemophiliac samples. Discrepant results in this multiply-exposed cohort might be explained by the detection of antibody to genotypes from previous or multiple infections

Finally, in an attempt to design a new serotyping assay, further subgenomic regions of RNA were cloned and expressed as recombinant protein for genotypes 1, 2 and 3. An ELISA using amino acids 1360-1454 of NS3 increased sensitivity for type-specific antibody, although cross reactivity between genotypes 2 and 3 was observed. To combine the sensitivity of NS3 with the type-specificity of the NS4 region, clones from these two regions were fused together and expressed as recombinant protein for a final prototype serotyping ELISA.

This work has enabled the specific identification of HCV serotypes causing infections worldwide by the development of a highly specific serotyping ELISA. Evidence concerning the possible biological differences between HCV genotypes suggests that serotyping may become an important method in clinical practice for the selection of patients for antivirus treatment.

AIMS

At the beginning of this work a competitive ELISA using type-specific peptides from the NS4 region had been described for the determination of HCV antibody serotypes 1, 2 and 3 (Simmonds *et al.,* 1993d). The aim of this thesis has been to extend this assay to include the more recently discovered genotypes 4, 5 and 6, and to validate it by measuring its sensitivity and specificity (see appendix 1.0) with different patient groups in various geographical populations.

Additional objectives were to improve the sensitivity and specificity of the assay, including the incorporation of additional antigens from the NS4 region. The ultimate aim of this work is the production of new serotyping assays based on several immunodominant regions, resulting in a highly sensitive ELISA which is able to detect and distinguish between the broad spectrum of genotypes present worldwide. Such an assay will be an important tool for epidemiological studies and possibly to help identify patients who are likely to show a good respose to antiviral therapy.

CHAPTER 1

1.1 GENERAL INTRODUCTION TO HEPATITIS C VIRUS.

1.1.1 NON-A NON-B HEPATITIS.

Despite the introduction in many countries of screening of blood donations for hepatitis B surface antigen in the 1970's, post-transfusion hepatitis continued to occur. Cases were diagnosed as non-A non-B hepatitis (NANBH) after the exclusion of infectious agents such as hepatitis A virus (HAV), hepatitis B virus (HBV) and members of the *Herpesviridae* such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) which were known to cause hepatitis in rare cases.

NANB hepatitis was generally mild in the acute phase with less than 50% of patients showing any clinical signs or symptoms, although a large proportion of infections progress to chronic hepatitis, and liver cirrhosis. The incubation period for post-transfusion NANBH of 8-10 weeks was consistent with an infectious source, although it was generally shorter than that for HBV infections and longer than that for HAV infections. This and other clinical evidence suggested that post-transfusion hepatitis was caused by an unidentified infectious agent (Feinstone *et al.,* 1975; Alter *et al.,* 1975; Prince *et al.,* 1974). Parenterally transmitted NANBH described above remains quite distinct from enteric NANBH, which is now classified as hepatitis E.

1.1.2 DISCOVERY OF HEPATITIS C VIRUS.

The causative agent of NANBH remained elusive for many years despite extensive research. The first advances came in 1979, when the disease was successfully transmitted into chimpanzees from a batch of factor VIII which had been associated with the infection of human recipients (Bradley *et al.*, 1979). In addition, it was found that the agent could be transmitted to other chimpanzees by the inoculation of plasma or liver preparations from a diseased animal in the acute-phase of infection. All infected animals developed liver histology characteristic of viral hepatitis, and all were negative upon serological testing for HAV, HBV, CMV and EBV. Further evidence that this agent was unrelated to HAV or HBV was the lack of protection by pre-existing antibody to these viruses in two of the chimpanzees. Possibly the most exciting result of this study was the identification by electron microscopy of 25-30nm virus-like particles from both factor VIII and the first passage chimpanzee liver biopsy by immune electron microscopy which was recognised using antibodies from a human patient. As these biopsy samples were consistently negative for HAV and HBV, these virus-like structures were identified as being the probable infectious agent causing NANBH.

Despite extensive studies using conventional immunological techniques, specific virus antigens or antibodies were not identified until 1989, when a cDNA library from chimpanzee plasma with a high infectivity titre was constructed into bacteriophage λgt11 (Choo *et al.*, 1989). Using serum from a patient with chronic NANBH as a source of antibody, the phage library was screened for clones

expressing virus antigen. This lead to the identification of an antibody binding clone designated 5-1-1. This clone had an open reading frame (ORF) which, when expressed in *E.coli*, resulted in a peptide that reacted positively with NANBH patient serum by Western blotting. To investigate the type of nucleic acid of the virus, serum was treated with either ribonuclease (RNase) or deoxyribonuclease (DNase) prior to hybridisation to the cDNA library. Hybridisation signals of nucleic acid complementary to the cDNA were lost after treatment with RNase but not DNase, leading to the conclusion that the infectious agent was a RNA virus. As only one of the strands of cloned cDNA could hybridise to the nucleic acid, (the strand complementary to that encoding the 5-1-1 ORF), the virus was identified as having a single stranded positive sense RNA genome and given the name hepatitis C virus (HCV).

Following this discovery, the first assay for the detection of HCV antibody was rapidly designed (Kuo *et al.,* 1989). The expression of three overlapping clones from 5-1-1 in yeast led to the synthesis of a 363 amino acid HCV polypeptide (C100-3), which was solubilised and purified before being coated onto the wells of a microtitre plate. The resulting ELISA was used to screen for HCV antibodies in a number of study groups, including blood donors and recipients from post-transfusion NANBH cases in the United States, Japan and Italy. It was discovered that up to 84% of Italian patients diagnosed with post-transfusion NANBH had antibody specific for HCV. Following these studies commercial assay systems were

developed, and the routine screening of blood donations for antibody to HCV was quickly introduced.

The first described HCV genome (figure 1.1.4), consisted of 9379 nucleotides in a single large ORF encoding a virus polyprotein precursor of 3011 amino acids (Choo *et al.,* 1991). A 5' non coding sequence was identified upstream of the ORF which showed similarity to that found in pestiviruses, and the polyprotein itself was related to both pestiviruses and flaviviruses. Comparison of partial sequences isolated from Japanese patients suggested the possibility of distinct virus genotypes (Enomoto *et al.,* 1990a; Kato *et al.,* 1990a).

1.1.3 CLASSIFICATION OF HCV AMONG OTHER RNA VIRUSES

Analysis of the first cloned nucleotide sequences of HCV revealed similarities to more than one existing virus family, making classification difficult. Although specific sequence motifs in certain nonstructural regions of the genome showed high levels of similarity with Dengue virus (a flavivirus), a greater level of protein sequence similarity was found with the animal pestiviruses bovine viral diarrhoea virus (BVDV) and Hog Cholera Virus (HChV), and to a lesser extent with certain picornavirus and plant viruses (Miller *et al.,* 1990). The recent re-classification of pestiviruses from the family *Togaviridae* into *Flaviviridae* based on genome organisation and structure (Collett *et al.,* 1988), led to the consensus that HCV should also be included in this family. Although members of both the *Flaviviridae* and *Picornaviridae* families possess a single large ORF like HCV, and

have a similar genome organisation with structural proteins at the 5' end and nonstructural genes nearer the 3' end, these families have different sizes of genomes and code for variable numbers of proteins. The *Flaviviridae* have many specific properties in common with HCV, including glycosylation of the envelope, and the cleavage of the polyprotein precursor into individual proteins by both virus encoded and host cell proteases.

Structurally, the HCV genome is similar to that of pestiviruses (BVDV and HChV) in both the arrangement of genes (Miller *et al.,* 1990) and the number of cleavage sites on the polyprotein, as well as physicochemical properties such as a low buoyant density in sucrose gradients (Miyamoto *et al.,* 1992). The HCV genome contains a long 5' non coding region (5'NCR), which shows remarkable similarity in secondary structure to other pestiviruses despite their divergent sequences, although quite distinct to that in flaviviruses for which a convincing secondary structure has yet to be proposed (Brown *et al.,* 1992).

For HCV and pestiviruses, the 5' NCR acts as an internal ribosomal entry site (IRES), and has been shown in picornaviruses to be essential in the initiation of cap-independent translation as an internal ribosome binding site. Flaviviruses on the other hand have no such structure, with the ribosome binding to a cap present upstream of the AUG initiation codon.

The current classification of HCV according to the International Committee on the Taxonomy of Viruses is as a separate genus in the *Flaviviridae* named the *Hepaciviruses*. Included in this genus are the newly discovered agents GB virus A

(GBV-A), GB virus-B (GBV-B) and GB virus-C (GBV-C), possibly misleadingly known as hepatitis G virus (HGV) (Linnen *et al.,* 1996; Simons *et al.,* 1995; Ohba *et al.,* 1996). These viruses all have genomes of similar sizes and organisation as the *Flaviviridae*, and are more closely related to HCV than other flaviviruses or pestiviruses (reviewed in Heringlake *et al.,* 1996).

Figure 1.1.4 THE HCV GENOME

Genome organisation of HCV, showing individual genes with the corresponding structural and non-structural proteins. Antigens used in HCV screening assays are also shown as boxes.

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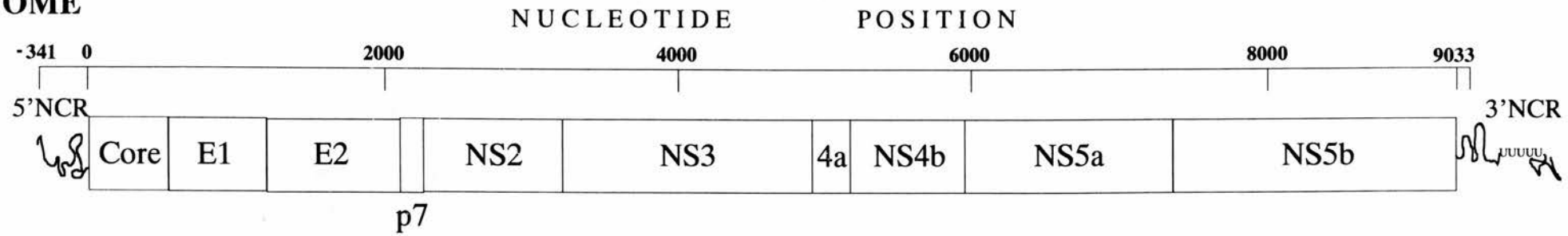
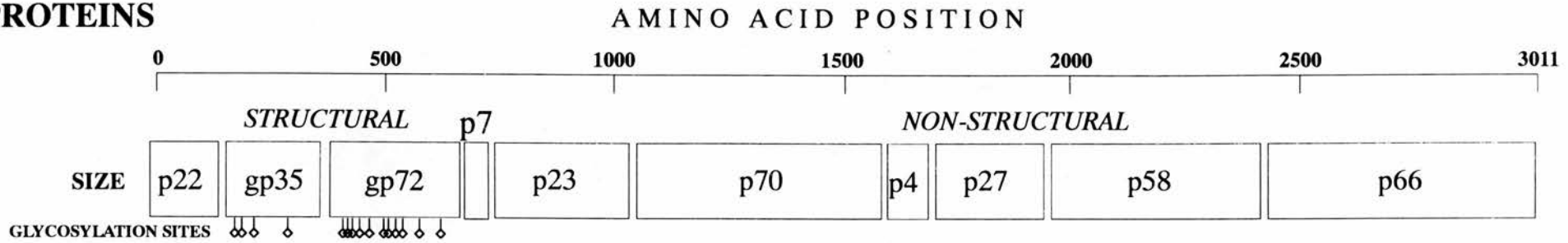
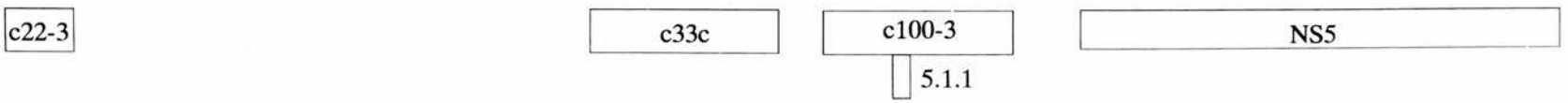
GENOME



PROTEINS



ANTIGENS



1.1.4 GENOME ORGANISATION

The genome of HCV is organised with genes coding for structural proteins positioned at the 5' end of the RNA, and non-structural (NS) protein coding regions towards the 3'end, in the order (5' to 3') core, envelope (E1 and E2), NS2, NS3, NS4a, NS4b, NS5a and NS5b (figure 1.1.4).

The 5'NCR is 341-344 bases in length and is highly conserved among different HCV genotypes. The secondary structure of this region has been shown to contain an internally base-paired stem loop, as in pestiviruses (Smith *et al.,* 1995b; Brown *et al.,* 1992b; Han *et al.,* 1991b), thought to function as an internal ribosome entry site (IRES) during translation (Wang *et al.,* 1993; Tsukiyama Kohara *et al.,* 1992). Several AUG codons occur within the 5'NCR, each followed by short ORF's, and it was hypothesised that the translational products from these may have a role in the regulation or initiation of translation (discussed in section 1.1.5). However, none of these ORF's are conserved between all genotypes of HCV, suggesting that their function, if any, is not essential for replication. A polypyrimidine tract between nucleotide positions 191 and 199 has been reported to play an important role in the initiation of translation (Yen *et al.,* 1995).

The 3' NCR consists of a relatively short sequence 200-235 nucleotides in length, with a 27-70 base type-specific region immediately following the termination codon on the polyprotein (Yamada *et al.,* 1996). Downstream of this region lies a poly A (Han *et al.,* 1991) or poly U (Yamada *et al.,* 1996; Kanai *et al.,* 1995; Tanaka *et al.,* 1995) tail of variable length (reports of between 10 and

100 residues), which was first thought to represent the end of the HCV genome. More recently, a C(U)n repeat has been identified immediately downstream of this region, followed by a highly conserved "core" element of 98 or 100 nucleotides capable of forming an internally base paired stem loop structure and terminated with a 3' OH group (Yamada *et al.,* 1996; Kolykhalov *et al.,* 1996; Tanaka *et al.,* 1995). Although the biological functions of the poly U tail, C(U)n repeat and the core element have still to be determined, it has been proposed that poly U binds part of the NS3 helicase (the function of which is discussed in section 1.1.5) (Kanai *et al.,* 1995), and that the high levels of conservation in the secondary structure of the core element implies an important role in the replication of the genome (section 1.1.5).

1.1.5 REPLICATION OF HCV

The lack of an efficient *in vitro* cell culture system for HCV has meant that description of the full replicative cycle of the virus remains limited. Full length HCV sequences have been successfully transfected as DNA into transformed human hepatoma cells (Yoo *et al.,* 1995), but this resulted in only very low levels of expression of virus proteins. In addition, *in vitro* replication of the HCV genome has been demonstrated in a murine retrovirus-infected human T-cell line (Shimizu *et al.,* 1992). The majority of information about HCV replication has resulted from the purification of virus encoded proteins, structural analysis of non-coding regions and comparisons with other positive stranded RNA viruses such as picornaviruses.

Transcription of the HCV Genome.

Based on the replication mechanisms of other single stranded positive sense RNA viruses, HCV is presumed to replicate via the synthesis of a complementary (antisense) RNA strand produced by an RdRp. Antisense RNA strands would then act as templates for the production of positive sense RNA. This theory has been supported by the detection of antisense RNA in the liver (Lanford *et al.*, 1994), and in rare cases in peripheral blood mononuclear cells (PBMC's) (Mellor *et al.*, 1998; Lerat *et al.*, 1996).

The subgenomic region encoding the RdRp was identified early on as NS5b, based on the high levels of sequence similarity with those present in other RNA viruses (Miller *et al.*, 1990). Using the 3' terminal residue of genomic RNA as a primer, the HCV RdRp has been shown to synthesise a complementary antisense RNA strand in a 3' to 5' direction using a "copy back" mechanism, producing a duplex RNA hairpin structure (Behrens *et al.*, 1996). The carboxyl terminus of NS3 has been shown to encode a virus helicase, which has nucleoside triphosphate activity and is thought to be involved in the unwinding of the duplex RNA intermediate during synthesis of positive sense RNA strands (Tai *et al.*, 1996). It has been hypothesised that the 3' NCR may play an important role in replication processes due to its highly conserved primary and secondary structure (Yamada *et al.*, 1996). However, clones of HCV lacking this region have been shown to replicate *in vitro*, albeit in low numbers, suggesting that such a role is nonessential

for replication, and may be responsible for regulatory aspects of the process (Yoo *et al.,* 1995; Tanaka *et al.,* 1995).

HCV Translation and Polyprotein Processing.

Translation of the HCV genome has been shown to be a cap-independent process initiated by the presence of an IRES in the 5' NCR (Wang *et al.,* 1993; Tsukiyama Kohara *et al.,* 1992). The extensive internal base-pairing of this region results in the formation of a specific secondary structure that is conserved between genotypes despite sequence variation at the primary level (Smith *et al.,* 1995b; Brown *et al.,* 1992b), and contains several stem loops which are thought to function as an IRES. Translation of the single large ORF is initiated at the methionine (AUG) codon at position 341, and although several small ORF's exist upstream of this region which were originally thought to express products involved in translation (Yoo *et al.,* 1992), it seems unlikely that they play an essential role as nucleotide sequence variation between genotypes at positions can replace up to five of these initiation codons (AUG) with those coding for alternative amino acids (Smith *et al.,* 1995b).

The single large ORF encodes a polyprotein of 3011 amino acids, which is then cleaved into at least 9 individual viral polypeptides by a combination of both host cell and viral proteinases (Grakoui *et al.,* 1993b). Structural proteins core (the nucleocapsid), E1 and E2 (envelope glycoproteins) are cleaved from the polyprotein precursor by a host signal peptidase located in the lumen of the endoplasmic

reticulum (Hijikata *et al.,* 1991b). Nonstructural proteins are cleaved by at least two virus encoded proteases. Cleavage of the NS2 / NS3 junction is performed by a zinc-dependent metalloprotease encoded by NS2 overlapping with the serine proteinase domain of NS3 (Hijikata *et al.,* 1993; Grakoui *et al.,* 1993). The remaining nonstructural proteins are processed by a serine protease encoded by the amino terminal region of NS3, which acts on junctions NS3/NS4a, NS4a/NS4b, NS4b/NS5a and NS5a/NS5b (Bartenschlager *et al.,* 1995a; Bartenschlager *et al.,* 1993a). X-ray crystallography has revealed that this enzyme is folded like a trypsin-like proteinase, and a structural zinc binding site (Love *et al.,* 1996). This enzyme forms a stable complex with the NS4a polypeptide (Kim *et al.,* 1997; Kim *et al.,* 1996), and is responsible for the cleavage of the NS3/NS4a, NS4a/NS4b and NS4b/NS5a junctions (Lin *et al.,* 1994b; Failla *et al.,* 1995b; Bartenschlager *et al.,* 1995b). The activity of this enzyme is dependent on the serine residue at position 1165, which is thought to represent the active site of the serine protease (Eckart *et al.,* 1993).

1.1.6 HCV PROTEINS.

STRUCTURAL PROTEINS

The core protein is 21-22 kDa in size and is thought to be a component of the virus nucleocapsid. It has been shown to associate with the membranes of endoplasmic reticulum in the cytoplasm *in vitro* and in transfected cells, and to

possess a highly basic RNA binding domain at the N-terminus of the polypeptide between amino acids 1 and 75 (Santolini *et al.,* 1994). The mechanism of assembly of these proteins with RNA into the nucleocapsid is unknown, although an interaction domain may be present at amino acids 82-102 (Nolandt *et al.,* 1997). The detection of a core-specific antigen from HCV purified from plasma of 26kDa, larger than the protein previously detected, suggests that core may be modified prior to nucleocapsid assembly (Takahashi *et al.,* 1992).

The HCV genome encodes two envelope proteins, E1 and E2. Each protein is thought to undergo extensive *N*-linked glycosylation after translation, resulting in glycoproteins 31-35 kDa (E1) and 68-72 kDa (E2) in size. During polyprotein processing, two precursors of E2 are produced; E2-NS2 and E2-p7. The cleavage of these products is carried out in the endoplasmic reticulum by a host signal peptidase, but this process is inefficient resulting in the production of both E2 alone and E2-p7 (Lin *et al.,* 1994a). The functions of either p7 or E2-p7 are unknown. Non-covalent interactions between E1 and E2 result in the formation of a complex (E1E2) that is highly immunogenic and capable of producing protective antibody in chimpanzees (Dubuisson *et al.,* 1994; Ralston *et al.,* 1993). Vaccination of chimpanzees with both envelope glycoproteins (expressed in a recombinant vaccinia virus expression vector) was able to prevent infection with HCV in 5/7 animals challenged with a homologous type 1 virus (Choo *et al.,* 1994). Despite these encouraging results, the development of a vaccine for HCV is hampered by the level of heterogeneity within HCV. Envelope proteins are highly variable in amino

acid sequence between genotypes of HCV, and a hypervariable region (HVR) in E2 may contribute virus persistence (section 1.2.5). Conserved elements of this region are several *N*-linked glycosylation sites and cysteine residues.

NONSTRUCTURAL PROTEINS.

NS2 is a hydrophobic 23kDa protein which is a zinc-dependent metalloprotease involved in cleavage of the polyprotein precursor (section 1.1.5). The HCV NS2 protein shows little sequence similarity with its equivalent protein found in *Pestiviruses* and *Flaviviruses*.

The NS3 region encodes a 70-72 kDa protein which functions both as a serine protease involved in polyprotein processing, and as an RNA helicase possessing NTPase activity (section 1.1.5). This is typical of several other *Flaviviruses*. All cleavage sites contain a cysteine or threonine residue (Lau *et al.*, 1994).

The 4kDa NS4a protein is a cofactor for efficient cleavage by the NS3 serine protease (section 1.1.5). The function of the 27kDa hydrophobic NS4b protein is unknown.

Both NS5a (56-58kDA) and NS5b (66kDa) are hydrophilic. NS5b is the virus RdRp (section 1.1.5), and although the function of NS5a has not been identified, a high level of sequence similarity with the equivalent gene in pestiviruses suggests that this protein may be involved in virus replication. In addition, recent evidence has demonstrated that NS5a associates with a cellular

serine/threonine kinase *in vivo*, and is phosphorylated on serine, and less frequently, threonine residues. This may indicate an important role for phosphorylated NS5a in the regulation of HCV replication processes, or perhaps a role for the cellular kinase during replication (Reed *et al.*, 1997).

1.1.7 HCV ANTIGENS

A wide range of both linear and conformational (dependent on secondary structure) epitopes have been identified within structural and non-structural regions of HCV. Many antigens have been expressed in either prokaryotic or eukaryotic systems as recombinant protein, or synthesised as short peptides and used in immunoassays.

HCV screening assays generally employ enzyme indicators to detect binding of antibody to a number of different virus antigens coupled to a solid phase (ELISA). A general explanation of the various types of ELISA used in this work is included in section 2.1.1. First generation assays consisted simply of the c100 antigen coated onto microtitre plates, and were successful in the identification of HCV infection in many blood donors, leading to a reduction in the incidence of post-transfusion hepatitis (van der Poel *et al.*, 1989; van der Poel *et al.*, 1990). The sensitivity of HCV screening assays was greatly increased (termed second generation assays) by the addition of antigens corresponding to part of the core protein (c22-3) and NS3 (c33c) (Takano *et al.*, 1996; Baath *et al.*, 1992; Aach *et al.*, 1991), to the existing c100 and 5-1-1 antigens. More recently, third generation

screening assays have been developed which include a further antigen corresponding to NS5 (Figure 1.1.4).

Other assays in the form of a recombinant immunoblot (RIBA) are also available for the serological detection of HCV infection, and the elucidation of the various antibody specificities that may be present in a sample. These consist of HCV antigens which have been immobilised onto nitrocellulose strips, and are generally more time consuming and expensive than the screening ELISA. As the antigens included in these assays are equivalent to those used in corresponding generations of screening ELISA, they are generally considered to be supplementary tests rather than confirmation tests. There is no serological method currently available for the confirmation of HCV infection.

NS4

The first HCV antigen to be used for the screening of blood donations was derived from the original HCV clone 5-1-1 (Choo *et al.,* 1989), and the subsequent analysis of several overlapping clones. This antigen, designated c100-3, was expressed in yeast and spanned a section of the NS3 region, as well as the majority of NS4a and NS4b (amino acids 1569-1931), and it or derivatives have been widely used in serological assays. Epitope mapping with synthetic peptides has revealed a number of linear epitopes in the NS4 region (Khudyakov *et al.,* 1995; Simmonds *et al.,* 1993), including amino acids 1691-1708, 1720-1738 and 1921-1940 (Figure 1.1.7). Many of these epitopes (for example residues 1691-1708 and 1720-1738)

differ sufficiently between genotypes of HCV to generate type-specific immune responses. Although this has been successfully exploited in the production of serotyping assays (section 1.5), this antigenic variation also means that the level of reactivity to these antigens in commercial serological assays, all of which contain antigen derived from HCV genotype 1, may vary with different genotypes (Dhaliwal *et al.,* 1996). The most recent assays (3rd generation) have replaced c100-3 fusion protein with synthetic peptides, and in doing so have reduced the genotype dependence of these assays.

CORE

The core protein is relatively conserved between genotypes of HCV, and often elicits a strong humoral response early during seroconversion (Sallberg *et al.,* 1992; Nasoff *et al.,* 1991). Epitope mapping of amino acids 1-190 using synthetic peptides, each 18 residues in length and overlapping the adjacent peptide by 8 residues, has revealed a number of linear epitopes in this region (Figure 1.1.7)(Sallberg *et al.,* 1992). A high level of reactivity from HCV positive samples was observed with peptides covering amino acid regions 1-38, 51-68 and 101-118. The most immunodominant region was between residues 1-28, with reactive antibody present in more than 90% infected individuals.

A recombinant protein (c22-3) covering a major section of the core region and expressed in yeast, is incorporated into the majority of second generation screening assays, resulting in an increase in sensitivity over the first generation

ELISA using c100-3 alone. Recently a 3rd generation supplementary immunoblot (RIBA 3.0) has replaced the recombinant antigen with synthetic peptides to amino acids 10-53 which has improved the specificity of the assay.

NS3

The NS3 protein is highly immunogenic, with an antibody response present in most established infections (Gussow *et al.,* 1990). Antibody to c33c is produced early during seroconversion, and is often the first to be detected by serological assays (Puoti *et al.,* 1992; Lelie *et al.,* 1992; Diepolder *et al.,* 1995). This antigen (c33c) was absent in most of the first generation assays but is included in all second and third generation assays and supplementary immunoblots. Immunoblot analysis has revealed four linear epitopes within the middle region of NS3 (Figure 1.1.7), around amino acids 1250/1251 and within residues 1250-1334 and 1407-1412 (Hwang *et al.,* 1996; Khudyakov *et al.,* 1995). However, the majority of epitopes in NS3 are conformational, having been recognised by monoclonal antibodies between residues 1363-1454 (Mondelli *et al.,* 1994), and by reactivity of HCV positive sera towards recombinant proteins representing amino acids 1359-1449 (Claeys *et al.,* 1995).

NS5

Epitope mapping with synthetic peptides has revealed a number of linear epitopes in NS5a, including amino acids 2271-2313 and 2381-2452 (Figure 1.1.7).

Relatively few epitopes have been described in NS5b, and those which have been identified in amino acid regions 2467-2486 and 2894-2913 are marginally immunoreactive (Khudyakov *et al.,* 1995; Yuki *et al.,* 1992).

Although an NS5 antigen was incorporated into the first generation assay by Wellcozyme/Murex, other manufacturers did not include this region until recently in 3rd generation tests. The NS5 recombinant protein was initially expressed in yeast, and included almost the whole protein (amino acids 2054-2995), but certain assays (Murex) have reduced the size of this antigen. The NS5 protein has not improved the specificity of screening assays, since indeterminate results due to reactivity to NS5 only have almost invariably proven negative by PCR (Dow *et al.,* 1996a).

1.2 EPIDEMIOLOGY AND CLINICAL FEATURES

1.2.1 PREVALENCE OF HCV

The prevalence of HCV infection in the general population is highly variable between different geographical areas, and is dependant upon many factors. It is difficult to calculate the prevalence of HCV in a population, as study groups may not reflect the general population as a whole. In most countries, the blood donor population is used as a reference, although in countries which adopt a "self exclusion" policy for donors likely to have been at risk of infection, this figure may underestimate the true prevalence of infection. In the U.K., the prevalence of HCV in the blood donor population is estimated at 0.015%, although the prevalence in organ donors may reflect a more accurate number at 1.08%, as this is an unscreened population (Wreghitt *et al.,* 1994). The lowest prevalence is observed in the "Western" world, in the United Kingdom, Scandinavia, Western Europe, North America and Australia, with a higher prevalence of infection in southern and eastern European countries. Screening of blood donors has estimated the HCV prevalence in China, Japan and Thailand to be 1-2%, with Middle Eastern countries such as Egypt and Central/Eastern Africa having the highest prevalence of HCV in the world, with up to 20% blood donors anti-HCV positive (Botte *et al.,* 1996).

1.2.2 ROUTES OF TRANSMISSION

HCV is transmitted through contact with infected blood or blood products, with perhaps the highest risk group being patients affected with genetic disorders such as haemophiliacs who have been treated extensively with clotting factors. As hundreds of donations contribute to each batch of clotting factor, the majority of these patients became infected with HCV (and other agents) until heat-inactivation procedures were widely introduced in 1985. Another blood product shown to transmit HCV infection is intravenous immunoglobulin. For example, two major outbreaks of HCV infection have recently been described, each from a single source of anti-D immunoglobulin given to rhesus negative pregnant women in 1977/78 (Widell *et al.,* 1997; Power *et al.,* 1995; Power *et al.,* 1994; Meisel *et al.,* 1995). Prior to the introduction of routine screening of blood donors in the early 1990's, one of the major recognised routes of transmission was blood transfusion.

Another major route of transmission since the 1960's (and in places where donor screening is practiced, the most frequent) is intravenous drug abuse (IVDA). The practice of sharing contaminated needles provides a rapid and efficient mode of transmission for HCV, and it is estimated that up to 80% IVD users may be infected (Westh *et al.,* 1993; Bolumar *et al.,* 1996). Poor sterile technique may also be strongly implicated in a high number of infections worldwide. These techniques include tattooing, piercing, acupuncture and particularly the use of unsterile needles in medical practice in countries such as Japan, Italy, Sweden and certain areas of the Third world.

It is thought that there is a low risk of horizontal and vertical (transplacental) transmission of HCV, although this may increase if an individual is co-infected with HIV, or if the level of HCV viraemia is particularly high (Ohto *et al.,* 1994). A low rate of mother to child transmission has been reported (5-15%) (Lam *et al.,* 1993; Wejstal *et al.,* 1992; Wejstal *et al.,* 1990; Degos *et al.,* 1991). Studies have been unable to determine wether transmission occurs in utero / during birth (vertical transmission) or by close contact within the family group (familial transmission). However, a greater number of children were found to be infected with HCV if both parents were HCV positive (10 of 23 studied), than children with an HCV positive mother only (2 of 10 studied) (Nishiguchi *et al.,* 1992), suggesting that inter-familial spread may also play a role in HCV transmission and that intra-uterine transmission is rare.

1.2.3 DISEASE PROGRESSION

The period between exposure to HCV and the onset of acute hepatitis is usually 7-8 weeks, after which virus RNA can usually be detected by PCR, and liver function tests such as the level of alanine aminotransferase (ALT) in sera may become abnormal. Seroconversion for antibody may not take place until months later. Acute hepatitis C is asymptomatic in 95% cases. However, clinical features may include fatigue, anorexia and the patients may become jaundiced. Although patients have been described in which acute hepatitis C has been followed by the

clearance of both virus RNA and eventually anti-HCV reactivity (Lelie *et al.*, 1992), a majority of patients develop chronic hepatitis.

The histological features of chronic hepatitis include the presence of lymphoid aggregates in portal tracts of the liver, vacuolization and ballooning of epithelial cells lining the bile ducts and peri-portal inflammation. The stages of disease have been classified into three major groups; (i) Chronic persistent hepatitis (CPH) or lobular hepatitis, which is observed in approximately 40% cases and is histologically defined by a lymphocytic inflammation in widened portal tracts, (ii) Chronic active hepatitis (CAH), also observed in approximately 40% cases and recognised by histological damage to all hepatic areas including portal, periportal and acinar areas, and (iii) CAH with liver cirrhosis in the remaining 20% cases. (reviewed in Lau *et al.*, 1995). The histological features of disease have also been related to the mode of transmission of the virus. Infections caused by exposure to high or repeated doses of virus such as blood transfusion or regular infusion of infected factor concentrates is associated with a higher incidence of chronic hepatitis and a more aggressive form of chronic liver disease than, for example, HCV infection contracted through intravenous drug abuse (Gordon *et al.*, 1993). The effect of lower doses is shown by the fact that only 50% women infected from a common source of anti-D showed evidence of persistent infection with HCV (Meisel *et al.*, 1995).

The progression of chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC) is slow, unless the patient is immunosuppressed, for example due

to co-infection with HIV, or immunosuppressive drug treatment following organ transplantation. Healthy patients with chronic HCV infection but normal ALT levels and limited histopathology have been reported, and may represent an asymptomatic carrier state of the disease (Prieto *et al.,* 1995). Chronic hepatitis C is generally progressive, and about 20% of patients develop cirrhosis after 20 years of infection, with liver failure a likely outcome. HCC typically develops only after 30 years of infection, usually in patients with cirrhosis. The mechanism by which HCV causes HCC is currently unknown, and was originally thought to result indirectly from extensive damage of liver cells. Recent reports, however, implicate the RNA helicase domain of NS3 which has been shown to possess DNA helicase activity *in vitro* and may affect cellular processes (Sakamuro *et al.,* 1995). In addition, NS3 is able to transform NIH3T3 cells *in vitro* (Sakamuro *et al.,* 1995). The treatment of patients with CAH with interferon-α is associated with a decrease or delay in the incidence of HCC, despite having no effect on ALT levels (Nishiguchi *et al.,* 1995).

Disease progression is also thought to be influenced by other host and virus related factors, such as age of acquisition (disease is more aggressive in patients infected later in life), virus genotype (section 1.4), and the duration of infection.

1.2.4 TREATMENT OF HEPATITIS C

The only therapy to date which is effective in the treatment of HCV infection is interferon-α (reviewed in (Hoofnagle *et al.,* 1997). However, the

success of this drug is limited. Only 50% patients show an initial response, and 50% of these relapse when the treatment is stopped. A long-term sustained response therefore occurs in approximately 25% patients treated. Response to interferon is indicated by normalisation of ALT levels, or more sensitively by the disappearance of HCV RNA by PCR. In sustained responders, virus RNA generally becomes undetectable 1-4 weeks after the onset of therapy and remains absent for at least 6 months after treatment, whereas virus RNA promptly returns in patients who relapse after treatment. Although the standard length of treatment was originally 6 months, further studies with extended periods of therapy (12-18 months) have usually resulted in a higher rate of sustained response (Craxi *et al.*, 1996). Interferon-α has also been used to treat patients with acute hepatitis, resulting in a significant reduction of patients progressing to chronic infection, and can be associated with a complete recovery (Lampertico *et al.*, 1994; Omata *et al.*, 1991).

Ribavirin, a nucleoside analogue with a broad spectrum of antiviral activity, has also been used to treat patients with chronic HCV infection. Although this drug is effective in decreasing viraemia and ALT levels, these effects are short lived after treatment is stopped (Di Bisceglie *et al.*, 1992).

Combination therapy with interferon-α and Ribavirin has given the most promising results to date, with a sustained response in 40% patients who had not responded or had only a short-term response to interferon-α alone (Brillanti *et al.*, 1994).

1.2.5 IMMUNE RESPONSE TO HCV

Although HCV causes persistent infection in the vast majority of patients evidence from immunocompromised patients suggests that the host immune system can suppress HCV infection to some extent, since in these patients progression of disease is more rapid than in the immunocompetent (Eyster *et al.,* 1993).

Both humoral and cellular immune responses follow infection with HCV. Antibody to non-structural proteins is thought to reflect active replication of the virus, while antibody to structural epitopes may be present in both current and cleared infections. IgM antibody production is not restricted to acute infection, and may also correspond to the level of disease activity as levels of anti-core IgM have been demonstrated to decline during treatment with interferon (Nagayama *et al.,* 1994).

The cellular immune response to HCV involves both CD4 (T helper cells) and CD8 T cells (Cytotoxic T lymphocytes / CTL's). CD8 T cells specific for epitopes from both structural (core and envelope) and non-structural (NS2, NS3, NS4 and NS5) proteins have been demonstrated by many groups, although there are conflicting ideas on how they relate to the course of infection (Battegay *et al.,* 1995; Ferrari *et al.,* 1994; Koziel *et al.,* 1993). A CD4 T cell response to core epitopes has been established with asymptomatic disease (Botarelli *et al.,* 1993), although in other patients this response correlates with progression to chronic infection, while those with self-limiting disease generally have CD4 T cells specific for NS3 more readily (Diepolder *et al.,* 1995). Five different CD4 T cell epitopes

have been identified on the core protein, and in 3 larger domains within E1, which are thought to represent the specific peptides processed by antigen presenting cells (e.g. macrophages) for the activation of CD4 T cell proliferation via major histocompatibility (MHC) class II molecules on the cell surface (Lechmann *et al.,* 1996). Interestingly, this study reported that the response of CD4 T cells towards presented virus peptides was inversely proportional to the level of B cell antibody response to non conformational antigens, and that this was often observed in patients who had cleared the virus. This was recently confirmed, in part, by Nelson *et al* (Nelson *et al.,* 1997) who have suggested that CD8 T cell activity also corresponded with a low level of viraemia, however a higher level of disease activity was noted in these patients.

The mechanism of persistence of HCV is poorly understood, although consensus leads to the idea of immune escape via selective mutation within epitopes in the envelope protein. Hypervariable regions present in the envelope genes mutate readily, and in doing so change the antigenicity of the protein allowing an escape from neutralising antibodies (Weiner *et al.,* 1992). This hypothesis is favoured by the observation that a higher rate of amino acid substitution within HVR-1 exists in immunocompetent patients, than in the immunocompromised host (Kato *et al.,* 1993; Kumar *et al.,* 1994). Another possible mechanism for the persistence of HCV may be virus interference with antigen presentation processes. A peptide, thought to have no stimulatory effect towards T helper cells, has been discovered within the core protein, which is able to bind to

MHC class II molecules, and in theory compete for this site with processed virus epitopes.

1.3 GENETIC HETEROGENEITY OF HCV

1.3.1 VARIATION OF DIFFERENT SUBGENOMIC REGIONS

The extent of nucleotide and amino acid sequence heterogeneity between different variants of HCV first became apparent when isolates from Japan were cloned and sequenced (Takamizawa *et al.,* 1991; Kato *et al.,* 1990) and showed 92% nucleotide sequence similarity with each other but only 79% similarity with the genome of the prototype virus from the U.S.A. (Choo *et al.,* 1991).

The level of heterogeneity between variants of HCV is not unlike that observed in other single stranded positive RNA viruses, for example polioviruses and coxsackie viruses, with as much as 34% divergence existing between complete genomic sequences (discussed in section 1.3.2). The amount of sequence and amino acid heterogeneity is not evenly distributed between different subgenomic regions, but is maintained over the genome as a whole. The levels of divergence in non-structural genes is relatively constant, but is lower in the 5' NCR and core, and greater in the envelope regions. Regions of sequence constraint generally exist where important biological processes require a specific amino acid motif or the presence of a specific secondary structure. Mutations in these regions are more likely to generate a defective virus which is unable to replicate and therefore survive.

The 5' NCR has a highly conserved secondary structure consisting of internally base-paired stem loops thought to function as an IRES for the initiation

of translation of the genome (Tsukiyama Kohara *et al.,* 1992; Brown *et al.,* 1992). Although limited sequence variation does occur between genotypes of HCV, the secondary structure remains relatively constant (Smith *et al.,* 1995b). Nucleotide substitutions, and in some variants single and double nucleotide insertions, occur in non-base-paired regions, and therefore do not alter the secondary structure of the RNA. In contrast, substitutions occurring within the base-paired stem of the loop are often covariant, so that internal bonding of stem loop structures is preserved.

A similar constraint on both sequence and amino acid heterogeneity is observed in the core region which is thought to code for the components of the virus nucleocapsid. Recent evidence suggests that the 5' end of the core region remains part of the IRES domain, and therefore important in translational processes (Reynolds *et al.,* 1996; Kamoshita *et al.,* 1997). This is supported by the observation of a highly conserved 5' end of the core protein, concluding a marked reduction in the frequency of synonymous substitutions compared with the rest of the genome (Smith *et al.,* 1997). A number of other highly conserved sequence motifs have been identified in core which are thought to be vital for the efficient replication of the virus. These include a possible RNA binding site represented by 10 residues of arginine and lysine between amino acid positions 39 and 62, and two nuclear localisation domains PRRGPR and PRGRRQP at positions 38-43 and 58-64 respectively (Bukh *et al.,* 1994).

Much higher levels of sequence variation exist in the regions coding for the envelope glycoproteins (E1 and E2) than in other parts of the genome, with up to

50% divergence between other HCV variants. One hypothesis is that amino acid substitution in these regions are promoted by "immune escape" of the virus, hence providing a mechanism of persistence in the host. An especially high mutation rate is observed at the amino terminus of E2 (amino acids 384-414) known as hypervariable region 1 (HVR1), which may contain epitopes that can neutralise the virus (section 1.2.5) (Weiner *et al.,* 1992).

1.3.2 HCV CLASSIFICATION INTO GENOTYPES

Until recently, the classification of HCV variants into genotypes led to confusion as research workers from various countries proposed a number of different systems of nomenclature. Initially, the variants were classified in their order of discovery, with the clones from the U.S.A. known as type I and those from Japan as type II. However, as more divergent variants were discovered, and their sequence similarity to existing viruses analysed more closely, this system has been reviewed (Simmonds *et al.,* 1994a; Simmonds *et al.,* 1993a). Phylogenetic analysis of either entire genomes or subgenomic sequences divides HCV variants into 6 major groups (genotypes), many of which could be further divided into subtypes. More than 31% divergence (i.e. less than 69% nucleotide sequence similarity) was observed between complete genomic sequences of different genotypes and 20-23% between subtypes. Variants with less than 9% sequence divergence are classified as isolates of the same virus subtype. Phylogenetic analysis of subgenomic regions, including core (Bukh *et al.,* 1994), E1 (Bukh *et al.,*

1993), NS4 (Bhattacherjee *et al.,* 1995) and NS5 (Simmonds *et al.,* 1993b), provides the same groupings of HCV variants as analysis of entire genomic sequences, leading to the conclusion that any of these regions of the genome could be used for classification.

Although the majority of new isolates are easily classified within the existing subtypes, recently discovered variants from South East Asia (Mizokami *et al.,* 1996; Mellor *et al.,* 1995; Tokita *et al.,* 1995; Tokita *et al.,* 1994; Apichartpiyakul *et al.,* 1994) have proven ambiguous. These isolates were initially classified as additional major genotypes and several subtypes, as sequence analysis revealed a higher level of genetic heterogeneity from any of the six existing genotypes than that defined by subtype boundaries (i.e. more than 23%). However, phylogenetic analysis demonstrated that these variants were more closely related to type 6a than to other genotypes, and could therefore be considered as divergent subtypes of type 6 (Mellor *et al.,* 1995a).

1.3.3 GEOGRAPHICAL DISTRIBUTION OF HCV GENOTYPES

The major genotypes, and certain subtypes of HCV show clear geographical differences in distribution. Types 1a, 1b, 2a, 2b and 3a cause infections over a broad spectrum of geographical areas, predominating in North America and Western Europe although they occur elsewhere at a lower frequency. Other genotypes are confined to specific regions. For example type 5 is rarely found outside of South Africa, and type 6a is generally found only in South East Asia.

Genotype 4 is mainly confined to Central and North Africa and the Middle East. Type 3 is responsible for the majority of HCV cases in Pakistan, Bangladesh and Eastern India, and also for a limited number of infections in a broad range of geographical areas.

Specific differences in geographical distribution have also been found between certain subtypes, for example, type 1a is most common in the United States, whereas type 1b is responsible for 73% HCV infections in Japan (Takada *et al.,* 1993), and type 1a infections in Japan are limited to haemophiliacs who have used clotting factor concentrates exported from the U.S.A (Okamoto *et al.,* 1992).

1.4 BIOLOGICAL IMPLICATIONS OF HCV GENOTYPES

1.4.1 DISEASE PROGRESSION

The implications of infection with different genotypes of HCV on the severity of disease and the rate of disease progression is currently an active but confusing area of research. Numerous publications describe conflicting conclusions regarding the effect of virus genotype on the level of viraemia during infection, ALT abnormalities, the development of chronic disease and the severity of liver damage.

Although it has been suggested that infection with genotype 1 is associated with a higher level of viraemia, and a more rapid progression of liver disease in patients with chronic HCV (Kobayashi *et al.,* 1996; Booth *et al.,* 1995), more recent evidence derived from assays equally sensitive for RNA, describes similar levels of HCV RNA in serum from patients infected with different virus genotypes (Hawkins *et al.,* 1997; Smith *et al.,* 1996; Zeuzem *et al.,* 1996; Lau *et al.,* 1996). Similarly, although ALT levels are elevated more frequently in individuals infected with type 1 than with type 2 (Prati *et al.,* 1996), no correlation between genotype and liver function tests was discovered in studies of either chronic HCV patients (Zeuzem *et al.,* 1996) or patients having undergone liver transplant (Zhou *et al.,* 1996).

There is a greater propensity for the development of chronic disease with type 1 infections compared to type 2 or 3 (Prati *et al.,* 1996; Feray *et al.,* 1995),

and this is also observed for liver transplant patients, for whom the exact duration of infection is known (Feray *et al.,* 1995). In this latter study at least, the argument that type 1 infections are often associated with a longer duration of infection (Pawlotsky *et al.,* 1995), and therefore a more advanced stage of disease, can be ruled out. Despite the observation that type 1 infected blood donors are more likely to progress to chronic disease than those infected with type 2 (Prati *et al.,* 1996), there was no correlation between genotype and the severity of liver damage in this, or a number of other studies (Benvegnu *et al.,* 1997; Zhou *et al.,* 1996; Zeuzem *et al.,* 1996; Prati *et al.,* 1996). On the other hand, there are a number of reports suggesting that a connection between genotype 1 infection and the development of cirrhosis / increased liver damage may exist (Nousbaum *et al.,* 1995; Feray *et al.,* 1995; Booth *et al.,* 1995; Kobayashi *et al.,* 1996; Gordon *et al.,* 1997).

Further research is clearly needed into this subject, taking account of factors that may independently influence the progression of disease, such as age, duration of infection, route of transmission and the level of viraemia. It remains possible that certain genotypes are particularly pathogenic. This may result from amino acid variation in proteins leading to a greater cytopathic effect on liver cells, and/or to more efficient mechanisms of immune escape. The observation that two hypervariable regions exist in HCV type 1b as opposed to one in other variants is consistent with either hypothesis (Kato *et al.,* 1992; Hijikata *et al.,* 1991).

1.4.2 DEVELOPMENT OF HEPATOCELLULAR CARCINOMA

Another active area of research involves the association between virus genotypes with the eventual development of HCC. The majority of relevant publications to date describe an association between type 1b infections and HCC, although many of these studies fail to consider additional epidemiological or host-related factors which may effect the conclusion of such studies, such as duration of infection and/or the prevalence of certain HCV genotypes in different geographical areas. Many research groups have analysed the genotype distribution among patients with HCC, and found a high percentage to be infected with type 1b (Kao *et al.,* 1995; Tanaka *et al.,* 1996; Haydon *et al.,* 1995). However, before any conclusions can be made from such study groups, one should first consider the relative prevalence of this genotype in the general population and other factors such as the duration of infection, age of acquisition and mode of transmission. Similar studies, which have attempted to control some of these parameters, have provided conflicting results. Analysis of cirrhotic patients with and without HCC in Taiwan, revealed a significantly higher percentage of type 1b infection in the HCC cohort (69%) compared to those without HCC (46%). Though the mean age of both groups of patients in this study was similar, the duration of infection of each could not be identified. Similar results were obtained from a study of Greek patients, where type 1b was identified as a risk factor associated with development of HCC even after the differences in age had been accounted for (Hatzakis *et al.,* 1996). Another study in the U.S.A. revealed that from 40 patients undergoing liver

transplantation, all of whom had end-stage liver disease, HCC had developed in 23% patients infected with type 1b, compared with just 3% those infected with other genotypes (mostly subtype 1a). Possibly the largest survey has been described by a collaboration of groups in Japan (Yamada *et al.,* 1995), in which a total of 4176 chronic HCV patients with various stages of liver disease were analysed. Patients infected with type 1 and type 2 had similar age distribution and histories of blood transfusion, and the percentage of type 1 infections in each disease category (e.g. chronic hepatitis, patients with cirrhosis) was similar at 69-76%. However, a 7% higher frequency of type 1 infections and a 7% lower frequency of type 2 infections was observed in patients progressing to HCC.

Only a few publications argue that HCV genotypes do not differ in their ability to cause HCC. Analysis of Japanese patients with and without cirrhosis, all of whom were infected by blood transfusion and had a similar duration of infection, revealed no significant difference in the number of type 1b infections in each group (Yotsuyanagi *et al.,* 1995). Indeed, one study from Korea identified a lower percentage of type 1b infections in HCC patients (60%) than in blood donors (80%) (Lee *et al.,* 1996). The HCV genotype distribution in HCC patients from Thailand corresponded to that in the general population (62.5% type 3), although only 11 patients were analysed (Songsivilai *et al.,* 1996).

Indisputable evidence regarding the relative abilities of different HCV genotypes to cause HCC will only become available from longitudinal studies

which control for the patients age at the time of infection, the route, dose and frequency of exposure and the duration of infection. Confirmation of a type-specific association with the likelihood of developing HCC may eventually be provided through the identification of the mechanism for cell transformation by HCV.

1.4.3 RESPONSE TO ANTIVIRAL THERAPY

There are numerous clinical studies of the host and virus related factors which may predict the response to interferon therapy. Characteristics associated with a sustained response to treatment, are a lower age and duration of infection (Hino *et al.,* 1994; Yoshioka *et al.,* 1992), the absence of cirrhosis prior to treatment (Craxi *et al.,* 1995; Tsubota *et al.,* 1994), a low level of viraemia prior to treatment (Le Guen *et al.,* 1997; Pawlotsky *et al.,* 1996; Martinot Peignoux *et al.,* 1995; Craxi *et al.,* 1995; Tsubota *et al.,* 1994; Hino *et al.,* 1994) and infection with a genotype other than type 1 (Zein *et al.,* 1996; Kanai *et al.,* 1995; Martinot Peignoux *et al.,* 1995; Craxi *et al.,* 1995; Tsubota *et al.,* 1994; Chemello *et al.,* 1994). The strongest evidence that infection with type 1b is associated with a lower rate of sustained response to interferon has come from several studies that control for other factors such as patient age, the duration of infection and the level of viraemia (Pawlotsky *et al.,* 1996; Martinot Peignoux *et al.,* 1995; Tsubota *et al.,* 1994). Multivariate analysis in such studies has revealed genotype to be a statistically independent factor associated with a response to treatment, having a

relative calculated risk of 16.23 compared to 5.43 for the presence of liver fibrosis and 3.56 for a high level of viraemia prior to treatment (Tsubota *et al.,* 1994).

Although the lower rate of response with type 1 is clear, the reasons why this should be so remain unknown. Little is known about the mode of action of interferon on clearing HCV infection, and the lack of an efficient cell culture system for the virus creates a giant hurdle for research into this subject. Interferon has been documented as having direct antiviral activity by the activation of target cells to synthesise effector proteins such as 2' 5'-oligo-adenylate synthetase (OAS). OAS polymerises ATP into 2' 5' oligoadenylates, thus activating a latent endoribonuclease which can destroy virus RNA during replication. However, interferon also has an immunomodulatory effect through enhanced expression of class I MHC antigens. Which of these mechanisms is involved in the action of interferon on HCV is not known, although reports of direct antiviral activity have been described (Grander *et al.,* 1996; Shimizu *et al.,* 1994; Brillanti *et al.,* 1991).

The lack of response to interferon of HCV type 1 infection is a relative, rather than an absolute phenomenon, and it is possible that amino acid substitutions in certain variants of type 1 create interferon resistant mutants. One group has recently suggested that unique amino acid substitutions in the carboxyl terminus of NS5a are connected with interferon resistance (Enomoto *et al.,* 1996; Enomoto *et al.,* 1995), however, these observations have not yet been reproduced by other research groups.

1.4.4 ANTIGENIC VARIATION BETWEEN GENOTYPES

It is recognised that a high level of amino acid variation exists between genotypes of HCV within certain immunodominant regions of the genome. The presence of type-specific antigens has important implications for both the efficient screening of blood prior to transfusion and the development of an effective vaccine. The lack of a cell culture system means that neutralisation studies for HCV cannot be carried out, although experiments with other viruses showing similar levels of genetic heterogeneity (e.g. serotypes of dengue virus) have revealed that the immune response generated against one virus type is unable to protect against infection with other serotypes.

Evidence suggesting that certain epitopes in HCV are type specific has been obtained from the analysis of reactivity towards recombinant protein or peptide antigens used in commercial screening enzyme immunoassays, all of which are derived from HCV type 1. It was discovered that only 32% sera from type 2 or 3 infections showed reactivity to the c100-3 (NS4) antigen in first generation assays, compared to 90% of type 1 sera (McOmish *et al.,* 1993). Similarly, reactivity of sera from non-type 1 infections with second generation assays was often limited to c22 (core) and c33c (NS3) antigens and absent from 5-1-1 and c100-3 (Zein *et al.,* 1995; McOmish *et al.,* 1994). Third generation screening assays use type 1 antigens derived from the core, NS3, NS4 and NS5 regions. A recent evaluation of reactivity levels in individuals infected by different genotypes, which also takes into account the level of viraemia and other host related factors, has revealed that

sera from blood donors with type 1 infections showed levels of reactivity more than four times greater than samples from individuals infected with type 2 or 3 (Dhaliwal *et al.,* 1996).

The presence of type-specific antigens in certain regions of the genome has led to the development of a number of different serotyping assays (section 1.5), and suggests that any future vaccine would have to be multivalent in order to protect against all known genotypes of HCV.

1.5 HCV TYPING ASSAYS

1.5.1 THE ROLE OF GENOTYPING

Different genotypes of HCV can be identified by the analysis of the sequence heterogeneity within subgenomic regions. The most reliable method involves the sequencing and phylogenetic analysis of amplified PCR products for coding regions of the genome. More rapid methods have also been described which allow the classification of HCV variants into genotypes and / or subtypes by the analysis of PCR products or through the detection of type-specific antibody (serotyping). These techniques play a central role in many aspects of HCV research, including epidemiological studies based on geographical differences in distribution and the efficiency of detection of different genotypes. HCV genotyping is also becoming increasingly important in clinical practice, with implications for disease progression and the prediction of response to antiviral therapy.

1.5.2 GENOTYPING METHODS

A number of different genotyping methods have been described which are able to type HCV variants because of type-specific sequences present in various subgenomic regions. The advantages and disadvantages of each method are summarised in table 1.5.2.

Amplification of PCR fragments using type-specific primers.

One of the first genotyping assays involved the amplification of core sequences with type-specific primers, designed so as to produce fragments of different length for genotypes 1a (I), 1b (II), 2a (III) and 2b (IV) (Okamoto *et al.*, 1992). This method involves two rounds of nested PCR, with universal primers in the first round, with the second round PCR using a universal sense primer and a mixture of four subtype-specific antisense primers directed to variable regions within core. The PCR products of different sizes were separated by gel electrophoresis. This assay was later modified to include primers specific for type 3 (Okamoto *et al.*, 1993), although problems associated with non-specific priming were encountered. The risk of non-specific amplification of products is greatly increased if sequence mismatches are present in the 3' end of the primers, and subsequent analysis of type 3 sequences revealed that 3 of the 5 terminal bases in this additional type 3 primer were also present in type 1a / 1b sequences. Further modification of primers and conditions (Widell *et al.*, 1994) has improved the assay to a certain extent, with fewer infections of mixed genotypes being obtained than with the original assay, which was often due to the non-specific amplification of genotypes.

A further modification of this assay has recently been described which uses type-specific primers in the core region for the identification of types 1a, 1b, 2a, 2b, 3a, 3b, 4, 5 and 6a (Ohno *et al.*, 1997). Although this assay claims to be more accurate than its predecessors, a limited number of types 4-6 were analysed, and

only 23 of 68 samples producing discrepant results between this assay and that described by Okamoto *et al* (Okamoto *et al.,* 1992) could be confirmed by sequencing.

Reverse-hybridisation to type-specific probes.

HCV genotypes can also be distinguished by a line-probe assay (LiPA), which involves the amplification of 5' NCR sequences with universal biotinylated primers, and the subsequent hybridisation of these PCR products to type-specific probes immobilised as parallel lines on a membrane strip. The first generation assay (Stuyver *et al.,* 1993) used probes directed to variable regions of the 5' NCR for types 1a, 1b, 2a, 2b, 3 and 4. The specificity of this assay was relatively high, with even single mismatches sufficient to prevent cross-hybridisation between types, and reactivity to multiple probes was only observed with genuine dual infections. Despite this, sequence similarity between certain subtypes led to misinterpretation in some cases (generally type 1 subtypes, and cross-hybridisation between types 1 and 4).

A second generation assay (Stuyver *et al.,* 1996b) has a total of 21 probes from 7 variable regions of the 5' NCR for the identification of types 1a, 1b, 2a/c, 2b, 3a, 5 and 6a. This version of the assay was highly specific for the majority of samples, although new problems were encountered as unusual HCV variants were discovered in South East Asia (now classified as additional subtypes of type 6)

which contained 5' NCR sequences identical to those of type 1b (Mellor *et al.*, 1995; Tokita *et al.*, 1995; Tokita *et al.*, 1994).

The misidentification of subtypes 1a and 1b in the first generation assay gave rise to the addition of probes directed to parts of the core region for both types 1 and 2 (Stuyver *et al.*, 1995), although this version of the assay was never commercially available. This increased the specificity of the assay for these subtypes, this assay remains unable to identify the novel type 6 subtypes.

Restriction fragment length polymorphism (RFLP).

Genotyping by this method involves the PCR amplification of sequences from the 5' NCR with universal primers, and the subsequent digestion of these products with specific restriction enzymes to reveal patterns of fragments with different sizes corresponding to each genotype. The assay was initially designed to distinguish between genotypes 1-3, then 1-6 (McOmish *et al.*, 1994), and was later extended to be able to discriminate between subtypes 1a/1b, 2a/2b and 3a/3b by means of an additional enzyme digestion step (Davidson *et al.*, 1995). Other variations of this method have been described (Murphy *et al.*, 1994), with the use of a number of different enzymes, and all have proven highly accurate. However, single base mutations within the 5' NCR may give rise to misleading results, and sequence identity in this region between type 6 subtypes and type 1 may have led to the misidentification of these variants in previous studies. This problem has been recently been addressed by Mellor *et al.* (Mellor *et al.*, 1996) who have developed

an RFLP assay for the core region which is able to distinguish between subtypes 1a, 1b and type 6 variants from S.E. Asia. While the use of this assay alone is not feasible due to the narrow range of subtypes identified, when used in combination with the 5' NCR RFLP it provides an accurate method for the identification of novel subtypes present in certain populations.

Other methods of genotyping.

Various other typing methods have been described, but which have been limited in the number of genotypes identified or in their sensitivity or specificity. A DNA enzyme immunoassay involving the hybridisation of amplified cDNA from the core region to type-specific probes attached to a solid phase (Viazov *et al.*, 1994) has been designed for the identification of types 1-3, and similarly a slot-blot hybridisation technique (Enomoto *et al.*, 1990b) using amplified NS5 sequences can distinguish between types 1 and 2, albeit with limited sensitivity.

1.5.3 SEROTYPING METHODS

The level of amino acid variation between genotypes of HCV is sufficient to cause certain epitopes to elicit a type-specific immune response, and as a result, a number of assays have been devised which are able to distinguish between different antibody serotypes. The majority of these are in the form of an ELISA, and involve the binding of HCV antibody present in serum to a type-specific antigen coated onto a solid phase (in the form of synthetic peptides or recombinant protein), followed by a second step in which bound HCV antibody is detected by the addition of secondary antibody (an anti-human antibody-enzyme conjugate). The principles and components of this assay are described in further detail in chapter 2.

Serotyping assays for types 1 and 2 have been developed using antigens from both the core and NS4 regions (Tanaka *et al.*, 1994; Machida *et al.*, 1992). An ELISA using synthetic peptides from the core region as antigen reported a highly specificity ("specificity" representing how accurately the assay has identified different HCV serotypes, see appendix 1.0) but a lower sensitivity ("sensitivity" representing the proportion of samples tested for which a serotype could be identified; see appendix 1.0) being able to detect antibody in only 68% of type 1 infections and 56% of type 2 infections. On the other hand, an assay based on recombinant proteins from NS4 (amino acid positions 1676-1760) was 98% sensitive when testing sera from patients with chronic hepatitis C in Japan (Tanaka *et al.*, 1994).

Recently, a serotyping method for the detection of patient antibody response to HCV types 1-3 has been developed in the form of an immunoblotting proceedure using a solid phase that incorporates peptides from both the NS4 and core regions (known as a RIBA strip). HCV serotype is inferred from the relative intensity of antibody reactivity towards the type-specific NS4 peptides. Reactivity to core peptides is used to analyse patients sera that are unreactive to NS4 (Dixit *et al.,* 1995).

All the above assays involve the direct binding of type-specific antibody to antigens on the solid phase, which involves a potential risk of non-specific binding by heterologous-type antibody (or any other non-specific binding of antibody). A different approach is to use type-specific NS4 peptides in a competitive ELISA (see chapter 2) for the detection of types 1-3 (Simmonds *et al.,* 1993d). In this assay, antigens specific to all genotypes are coated onto the solid phase, and antibody serotypes detected by the co-incubation of a competing solution of type-heterologous peptides with the patient's serum. Homologous type antibody is not blocked by the competing peptides and is therefore able to bind to the antigen on the solid phase.

Table 1.5.2 ADVANTAGES AND DISADVANTAGES OF DIFFERENT GENOTYPING METHODS

Method	Types Detected	Region	Advantages	Disadvantages	Reference
Type-specific primers	1a/1b 2a/2b 3a	Core	Rapid, inexpensive Does not mis-identify type 6 group variants	Misidentification of mixed infections due to non-specific primers amplification of genotypes	Widell *et al.* 1994 Okamoto *et al.* 1993 Okamoto *et al.* 1992
Type-specific primers	1a/1b, 2a/2b, 3a/3b, 4, 5, 6	Core	Rapid, inexpensive, Does not misidentify type 6 group variants	Performance of assay for types 4-6 unknown	Ohno *et al.*, 1997
Hybridisation	1a, 1b, 2b/c, 2a, 3a, 4, 5, 6a	5'NCR	Accurate for genotypes in Western countries	Expensive. Misidentification of type 6 group variants. Subtypes cannot be accurately identified	Stuyver *et al.* 1996 Stuyver *et al.* 1993
RFLP	1a, 1b, 2a, 2b/c 3a, 3b, 4, 5, 6a	5'NCR	Accurate for identification of major genotypes	Misidentification of type 6 group variants. Subtypes cannot be accurately identified	Davidson *et al.*, 1995 McOmish *et al.* 1994 Murphy *et al.*, 1994
RFLP	1a, 1b, type 6 group variants	Core	Identification of type 6 group variants.	Limited number of genotypes detected	Mellor *et al.*, 1996
DNA enzyme immunoassay	1a, 1b, 2a, 2b 3a	Core	Convenient. Reliable identification of common subtypes	Limited range of genotypes detected	Viazov *et al.* 1994

ELISA (peptides)	1-6	NS4	Accurate. Rapid. Easy to perform. Competition format increases specificity	Subtypes not detected. Problems with seronegative individuals	Bhattacherjee *et al.* 1995 Simmonds *et al.* 1993
ELISA (peptides)	1, 2, 3	NS4, Core	Rapid. Easy to perform.	Subtypes not detected. Problems with seronegative individuals. Limited range of genotypes detected.	Dixit *et al.* 1995
ELISA (peptides)	1, 2	Core	Rapid. Easy to perform.	Subtypes not detected. Problems with seronegative individuals. Limited range of genotypes detected.	Machida *et al.* 1992
ELISA (Recombinant protein)	1, 2	Core	Rapid. Easy to perform	Subtypes not detected. Problems with seronegative individuals. Limited range of genotypes detected.	Tanaka *et al.* 1994

CHAPTER 2

2.1 GENERAL INTRODUCTION TO ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA'S)

2.1.1 IMMUNOASSAYS

An immunoassay is an assay that uses antigens for the determination of specific antibodies. A number of different types of immunoassay have been developed, which use various methods for the detection of antibody. The most common methods include radioimmunoassays (RIE), which use radioisotopes as a label and determine the presence of antibody by scintillation counting, and flouroimmunoassays (FIE), which are flourimetric assays that use a flourescent compound as a label. Perhaps the most common type of immunoassay is the enzyme immunoassay (EIA). These assays are mostly colourimetric, and reveal the presence of specific antibody through an enzyme label, which upon addition of the appropriate substrate compound will display a colour which can be recognised by eye and measured accurately by spectrophometry.

The term ELISA is used for all kinds of EIA, and does not indicate a particular assay mechanism.

2.1.2 PRINCIPLES OF ELISA

Direct / Indirect ELISA's

The difference between direct and indirect assays lies in the component under detection. An indirect ELISA is used to screen for specific antibodies via capture by antigen that is coated onto the solid phase. A direct ELISA is used for the detection of soluble antigens in serum. Microtitre plates are coated with antigen, and enzyme labelled antibody (specific to the antigen on the plate) is prevented from binding if free antigen is present in the sample.

The basic principles of an indirect ELISA (used throughout this work) are shown in figure 2.1.2. Antigen (specific to the antibody under detection) is synthesised and coated onto the solid phase. In this work the antigen used consists either of branched peptides or recombinant protein. Incubation of a sample containing specific antibody for this antigen will result in the binding of the antibody to the antigen on the solid phase. After a washing step, bound antibody is detected via a secondary antibody (conjugate) which is selected to recognise the type of antibody under detection (IgG, IgM etc) and has been labelled with an enzyme. Examples of enzymes commonly used are horseradish peroxidase (HRP) and alkaline phosphatase. The enzyme label produces a colourimetric reaction upon incubation with a particular substrate compound. The two most sensitive substrates used with HRP are o-phenylenediamine (OPD) and tetramethylbenzidine hydrochloride (TMB), with both compounds being able to detect low levels of

Figure 2.1.2. Basic principles of an indirect ELISA

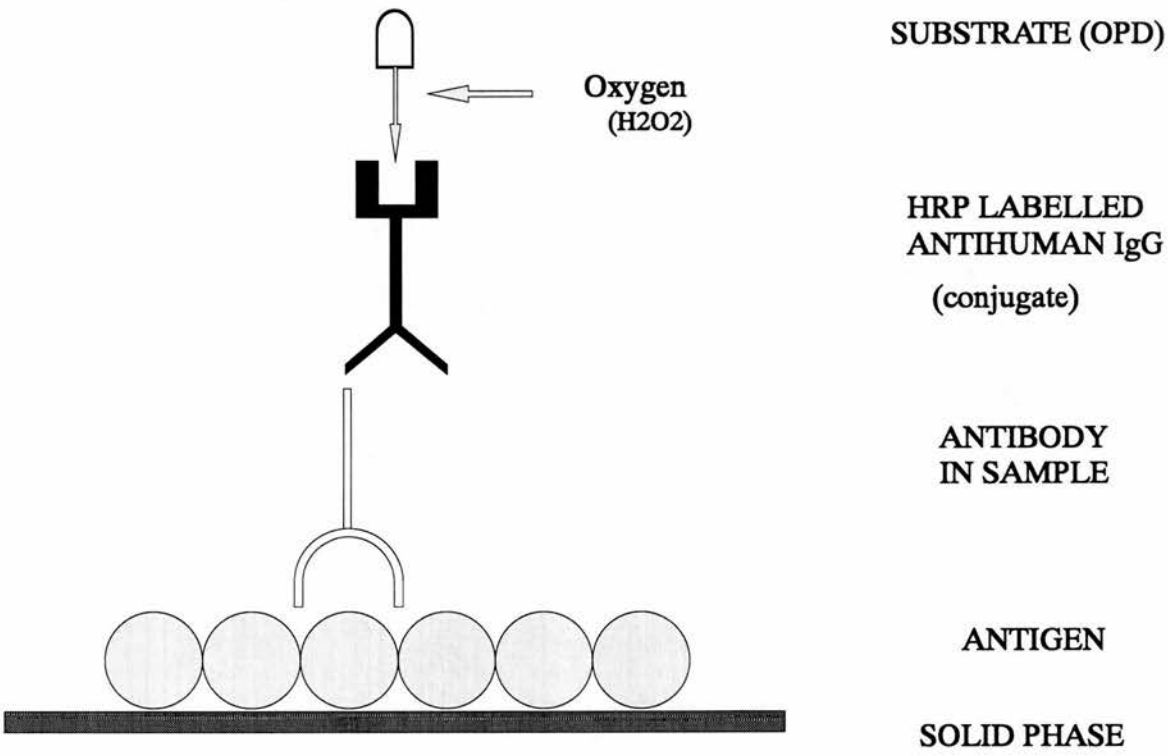


Figure 2.1.2 Basic principles of an indirect ELISA.

HRP - Horseradish peroxidase enzyme
OPD - o-phenylenediamine substrate

enzyme. The reactivity of OPD with HRP requires the presence of a catalyst which provides oxygen ions such as hydrogen peroxide.

Competitive assays

Many types of competitive ELISA have been developed, including those which compete for antigen and those which compete for antibody. The competitive nature of the serotyping assay used throughout this work involves the coincubation of type-specific antigen with NS4 antibody in the sample. If antigen in the competing solution is specific to the same genotype as the antibody present in the sample, it will effectively block this antibody and prevent binding to antigen on the solid phase. However, if only competing antigens specific to heterologous genotypes are present in solution, the antibody present in the sample will remain free and able to bind to the solid phase antigen. The serotyping assay incorporates various different combinations of competing antigens specific to different genotypes in order to differentiate between the different types of antibody.

2.2 ELISA's FOR HCV ANALYSIS

2.2.1 HCV IgG ANTIBODY DETECTION

Serum samples were tested for the presence of anti-HCV IgG antibody with the Murex anti-HCV enzyme immunoassay kit (VK47/48) according to the

manufacturers protocol. Briefly, 20μl serum was diluted 1:10 in sample diluent and incubated for 1 hour at 37°C in microtitre wells coated with antigens derived from the core, NS3, NS4 and NS5 regions of HCV. The plate was washed 5 times with glycine/borate solution prior to the addition of 100μl horseradish peroxidase-labelled mouse anti-human IgG conjugate. After 30 minutes at 37°C, the wash procedure is repeated, and 100μl TMB substrate solution was added and the plates were incubated for 30 minutes at 37°C. Reactions were stopped by the addition of 50μl 2M sulphuric acid and the absorbance of each well at 450nm was read within 15 minutes.

2.2.2 HCV SEROTYPING ASSAY FOR TYPES 1-3

Optimisation experiments for the serotyping assay (e.g. concentrations of antigens coating plates and in competing solutions) were carried out during the development of this ELISA (prior to this work) by Mr Ken Rose (Simmonds *et al.,* 1993d).

Preparation of Immunoplates

NS4 peptides were dissolved in AnalaR water (BDH) at a concentration of 1mg/ml. Insoluble peptides were first dissolved in 25μl of 30% acetic acid, and then made up to a concentration of 1mg/ml with distilled water (type 3 peptides), or suspended in distilled water by vortexing and bubbling through approximately 0.5ml ammonia vapour.

Stock peptide solutions were stored at 4°C for up to two weeks, but not frozen to avoid precipitation of the peptide. 96 well flat-bottomed microtitre plates

(NUNC Maxisorp, GIBCO/BRL) were coated with 100μl per well of phosphate buffered saline (PBS, SIGMA) containing eight NS4 peptides corresponding to two antigenic regions for HCV types 1, 2 and 3, each at a concentration of 50ng/ml (5ng / well) and incubated at 4° C overnight. Columns 1 and 7 were not coated and served as controls for non-specific binding to the solid phase.

Plates were washed 5 times with PBST (PBS containing 0.05% Tween 20 (BDH)) and air dried. Plates could be stored at 4° C for several months without a loss in reactivity.

Competing peptide solutions.

The serotyping assay relies on the complete blocking of antibody binding to epitopes that are shared between more than one genotype, therefore solutions containing different NS4 peptide combinations at the empirically derived concentration of 100 fold excess that used for coating the plates were added to the wells prior to the addition of serum.. Competing solutions containing only heterologous peptides (i.e. those different from that which the typing well is to detect) were added to each of the three typing wells, as well as an additional well with competing peptides for all genotypes present ("fully blocked" negative control) (Figure 2.2.3). Competing solutions contained peptides at a concentration of 500ng/well, i.e. 100 times the coating concentration.

Assay Procedure

Immediately prior to use, plates were coated with 125μl PBS containing 2% bovine serum albumin (BSA, SIGMA) for 1 hour at room temperature, in order to prevent the non-specific binding of antibody to any remaining protein binding sites in the plastic wells, after which the plates were washed in PBS.

Each test serum was diluted 1/40 with PBST containing 2% BSA (a concentration which gave high absorbence readings in combination with low levels of background reactivity when the assay was initially developed), and 100μl volumes added to each of the six test wells (uncoated negative control, unblocked positive control, fully blocked negative control and typing wells 1, 2 and 3). Plates were sealed and incubated at 37°C for 1 hour. After washing 5 times with PBST, 100μl of anti-human IgG HRP conjugate (SIGMA) diluted 1:20000 in 2% BSA in PBST, was added to each well. After incubation at 37°C for 1 hour, the washing step was repeated prior to the addition of 100μl per well of o-phenylenediamine (OPD) substrate (Appendix 2.2) and incubation of the plates for 30 minutes at room temperature in the dark. The colourimetric reaction was stopped with 50μl 8M sulphuric acid, and the plates read against an uncoated well negative control at a wavelength of 450nm. Samples showing relatively weak reactivity (i.e. those containing only low concentrations of HCV antibody) could be retested using higher or lower dilutions of serum in order to give a clear result.

Calculation of results

The serotype of HCV antibody in a sample can, in most cases, be identified by eye, as the assay involves a colourmetric reaction (section 2.1). However, conclusive identification of HCV antibody serotype transpires only if the absorbance values meet the following criteria (formulated by Murex Biotech Ltd for the commercialisation of this assay);

(1) The absorbance value in the "unblocked control" well minus the absorbance value in the "fully blocked" control well is greater than or equal to 0.1.

(2) The highest absorbance value (from the typing wells) divided by the absorbance value in the "unblocked control" well is greater than or equal to 0.4.

In general, the "unblocked control" should give the highest absorbance value as this represents the reactivity of all HCV antibody in the sample which is able to react with the combination of type-specific antigens on the solid phase. The "fully blocked" control contains competing peptides specific to all HCV genotypes being tested, resulting in the binding of only non-specific NS4 antibody to the solid phase and therefore a low absorbance value. Criterion (1) represents the cutoff value for reactivity, by calculation of the level of reactivity of type specific antibody. Criterion (2) represents the cutoff value for which antibody reactivity in the typing wells can be considered indicative of the presence of HCV type specific antibody. Although failure of both criteria means that the sample is untypeable, if criterion (2) only is not met and has only just failed (e.g. typing well / unblocked control = 0.37), the sample may contain very low levels of type-specific antibody and

should therefore be retested at a higher concentration. Similarly, if a high level of background reactivity is present, then criterion (2) may be met by more than one (or all) of the typing wells. In this case the absorbance value in the fully blocked control should be subtracted from the absorbance values and both criteria recalculated. If more than one typing well meets criterion (2) with a low level of background reactivity, the sample is considered to contain HCV antibody to more than one serotype, representing a multiple infection.

2.2.3 HCV SEROTYPING ASSAY FOR TYPES 1-6

Methods were identical to those for the 1-3 assay with the following exceptions.

Preparation of Immunoplates

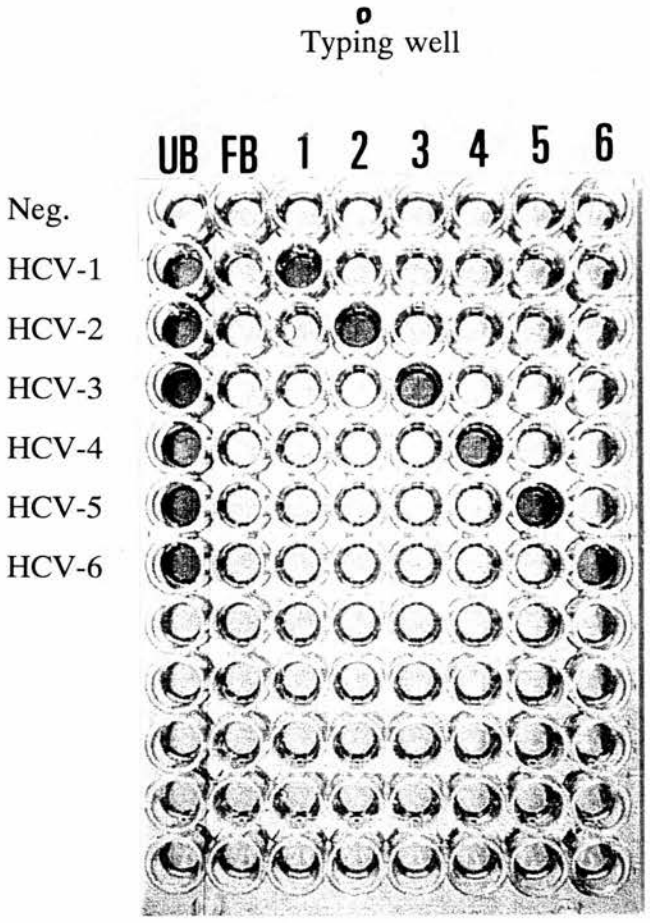
The uncoated negative control well was omitted (BSA had been shown to be an effective blocking agent against non specific binding of antibody) to fit the maximum number of test samples on one plate, and so all 96 wells were coated with antigen, and one column of 8 wells used per sample (unblocked positive control, fully blocked negative control and typing wells 1 through 6). All the peptides for genotypes 4, 5 and 6 were soluble in ammonia vapour.

Competing solutions consisting of heterologous peptides only were added to each typing well as shown in figure 2.2.3. The "fully blocking" competing solution contained 100μl of each of 21 peptides.

Figure 2.2.3 COMBINATIONS OF NS4 PEPTIDES USED IN COMPETING SOLUTIONS FOR THE 1-6 SEROTYPING ASSAY

		Type-specific peptides					
		1	2	3	4	5	6
	Fully blocked	✔	✔	✔	✔	✔	✔
Competing solution	1	✖	✔	✔	✔	✔	✔
	2	✔	✖	✔	✔	✔	✔
	3	✔	✔	✖	✔	✔	✔
	4	✔	✔	✔	✖	✔	✔
	5	✔	✔	✔	✔	✖	✔
	6	✔	✔	✔	✔	✔	✖

Figure 2.2.4 MICROTITRE PLATE SHOWING POSITIVE REACTIVITY FOR HCV TYPES 1-6 USING THE 1-6 SEROTYPING ASSAY



A positive reaction is indicated by a blue colour in the well of the microtitre plate.

UB Unblocked positive control well

FB Fully blocked negative control well

HCV serotyping assay for genotypes 1-6 (Murex Biotech)

After the development of the 1-6 serotyping assay, a commercial kit was produced by Murex Biotech. The procedure for this assay was identical for the "in house" assay except that samples were diluted 1/40 in a total volume of 200μl sample diluent per well, and the substrate, TMB, was mixed with an equal volume of tri-sodium citrate / hydrogen peroxide solution. A colour change from pink to blue is observed during a positive reaction.

2.3 THE POLYMERASE CHAIN REACTION (PCR)

2.3.1 EXTRACTION OF VIRUS RNA

In order to avoid degradation of RNA by nucleases, all solutions used in the purification of virus RNA were made up with distilled water treated with diethylpyrocarbonate (DEPC) at 0.05% for a minimum of 2 hours before being autoclaved, and gloves were worn at all times

Stock solutions of TNE buffer + 0.5% SDS + 10% (0.11M NaCl; 55mM Tris pH 8.0; 1.1mM EDTA pH 8.0; 0.55% SDS), proteinase K (Boehringer) (10mg/ml) and RNA buffer / carrier poly-adenylic acid (poly A; Pharmacia) (2mg/ml) were prepared. Extraction buffer was made by adding 9 volumes TNE buffer, to 1 volume of proteinase K and 20μl poly A, thereby giving a working solution of 0.1M NaCl; 50mM Tris pH 8.0; 1mM EDTA pH 8.0; 0.5% SDS, with

1mg/ml proteinase K and 40μg/ml poly A. This solution was incubated at 37° C for 10 minutes to allow the degradation of nucleases present in the solution.

Next, 100μl serum was added to 400μl extraction buffer in a 1.5ml eppendorf tube, mixed thoroughly on a vortex mixer and then incubated in a 37° C water bath for 1.5 - 2 hours. RNA was extracted by the addition of 450μl water-saturated phenol (Rathburn Chemicals) to each tube, which were then placed on a shaker at 300 revs / second for 5 minutes. The aqueous phase was separated by centrifugation at 15,000 rpm for 5 minutes, leaving unwanted cellular proteins in the bottom layer (phenol).The top aqueous layer containing nucleic acids was added to a new eppendorf containing 450μl chloroform / isoamyl alcohol (50:1). After mixing / centrifuging as before, the aqueous layer was transferred to a new eppendorf tube containing 40μl 3M sodium acetate pH 5.2, and an equal volume of 100% ethanol added prior to incubating at -20° C overnight to precipitate the nucleic acids.

Nucleic acids were pelleted by centrifugation at 15,000 rpm for 10 minutes at 0° C, washed by adding 600μl 80% ethanol (kept at 0° C). and recentrifuged. The nucleic acid pellet was dried on a hot block at 42° C for 10 - 15 minutes prior to re-dissolving in 25μl DEPC treated water.

2.3.2 REVERSE TRANSCRIPTION OF VIRUS RNA

In order to copy virus RNA into complementary DNA (cDNA), 5μl nucleic acid dissolved in DEPC water was added to buffer containing 50mM Tris-HCl pH

8.0; 5mM $MgCl_2$; 5mM DTT; 50mM KCl; 0.05μg/ml BSA, and 600μM of each of dGTP, dATP, dTTP and dCTP, with 20% DMSO, 1.5μM primer (outer antisense; Table 2.3), 10 units reverse transcriptase (RT, Promega) and 5 units RNAsin (RNase inhibitor - Promega), in a final volume of 20μl made up with DEPC water. The mixture was then incubated at 42°C on a hot block for 30 minutes.

In the majority of this work, the primer used for reverse transcription was the outer antisense PCR primer, but where only small sample volumes were available, less efficient random priming by hexameric oligonucleotides was chosen.

2.3.3 PCR AMPLIFICATION OF DNA

Two rounds of nested PCR were performed for each sample; a primary PCR reaction using "outer" nested primers which were positioned outside the desired region, and a secondary reaction using "inner" nested primers flanking the sequence of DNA to be amplified. The nucleotide sequences of primers used in this work is shown in Table 2.3.

For primary PCR, 5μl cDNA was made up to a final volume of 50μl with 1x PCR buffer with 1.5mM $MgCl_2$ (10X stock, Promega), containing 33μM each of dGTP, dATP, dTTP and dCTP, 0.5μM of each of the outer primers and 20 units/ ml *Taq* DNA polymerase. A layer of liquid paraffin was added to prevent evaporation, and the tube placed on a thermal cycler for 25 - 30 heat cycles

Table 2.3 HCV Primers

Primer	Orientation	Genotypes	Primer Sequence (5'→3')	Reference
5'NCR				
209	I/AS	1-6	ATACTCGAGGTGCACGGTCTACGAGACCT	*
939	I/S	1-6	CTGTGAGGAACTACTGTCTT	*
211	O/AS	1-6	CACTCTCGAGCACCCTATCAGGCAGT	*
940	O/S	1-6	TTCACGCAGAAAGCGTCTAG	*
Core				
410	I/AS	1-6	ATGTACCCCATGAGGTCGGC	†
954	I/S	1-6	ACTGCCTGATAGGGTGCTTGCGAG	†
951	O/AS	1-6	CAGGTRAGGGTATCGATGAC	†
953	O/S	1-6	AGGTCTCGTAGACCGTGCATCATG	†
NS3				
753	O/AS	1-6	GCGTCATAGCACTCACAGAGGAC	‡
751	O/S	1-6	TTYCGGGCNGCYGTGTGCACC	‡
593	I/AS	1-6	CACAAGCTTARCAGTCKATSACNGARTC	‡
594	I/S	1-6	ACCRCTGCAGAYCCYAACATNGAGGARGT	‡
593-T6	I/AS	6	CACAAGCTTAACAGTCGATGACAGAATC	This work
594-T6	I/S	6	ACCRCTGCAGATCCTAACATAACAGAGAC	"
LP8	I/S	1+2	GCGGATCCCTACMSNAACATMGAGGAGGT	"
LP9	I/S	3	GCGGATCCCTATYCTAACATCGAAGAAGT	"
LP10	I/AS	1-3	CCACTCGAGRCAGTCKATSACNGAGTC	"
NS4				
007	O/AS	1-3	AACTCGAGTATCCCACTGATGAAGTTCCACAT	¶
220	I/AS	1-3	CACATGTGCTTCGCCCAGAA	¶
53510	O/S	1-4	TTTTGGATCCATGCATGTCAGCTGATCTGG	¶
5668	O/S	1	ATGCATGTCRGCTGAYCTGGA	This work
865	O/S	6	CTGGAGGTTATCACNAGCACNTGG	"
866	I/S	6	RTCCTCGTGGGTGGAGTCCTNGC	"
867	AS	6	TTCCACATRTGYTTNKSCCAGAA	"
LP2	O/AS	1	GTACTGTATCCCGCTGATGAARTTCCACA	"
LP3	I/AS	1	TTCCACATGTGYTTCGCCCAGA	"
LP4	O/AS	3	GTATTGGATCCCACTCACAAAATTCCACA	"
LP5	I/AS	3	TTCCACATATGCTTGTGCCAGA	"
LP6	I/S	1	CTGGAGGTCGTCACTAGCACCTGG	"
LP7	I/S	3	CTGGAAGTAACCACCAGCACCTGG	"
LP11	I/S	1	GCTCTCGAGCCYGACAGGGARGTYCTCTAC	"
LP12	I/S	2	GCTCTCGAGCCSGACAAGGARRTCYTNTAT	"
LP13	I/S	3	GCTCTCGAGCCAGACAAAGAGGTGTTGTAT	"
LP14	I/AS	1	CAAAGCTTAGAAYTGCTCGGCGAGCWKCATYCC	"
LP15	I/AS	2	CAAAGCTTANAGCATCTCSGCNATCCGCTGCCC	"
LP16	I/AS	3	CAAAGCTTAGAACTGGTGGGCTATTACCTGAGC	"
pRSET				
d632	S		CATGGTATGGCTAGCATGAC	§
d662	AS		TATTGCTCAGCGGTGGCA	§
pTAG				
8819	S		GCTATGACCATGATTACGCCAA	§
9130	AS		ACACGTGTGGTCTAGAGC	§

* Chan *et al.*, 1992.
† Mellor *et al.*, 1995.
‡ Dr. V. Bhattacherjee; Personal Communication
¶ Simmonds *et al.*, 1993.
§ d632 / d662 were obtained from Murex Biotech Ltd, Dartford, U.K.
8819 / 9130 were designed by Dr. C Blake, University of Edinburgh.
Restriction sites are underlined

For secondary PCR, a 1μl aliquot from the primary PCR reaction was transferred to a tube containing 19μl PCR buffer containing inner nested primers and cycled as before.

2.3.4 ANALYSIS OF PCR PRODUCTS BY GEL ELECTROPHORESIS.

20μl amplified PCR product was loaded onto a gel containing 2% agarose in 1x TBE (89 mM Tris borate; 89 mM boric acid; 2 mM EDTA) and ethidium bromide at 0.05μg/ml. Electrophoresis was carried out in 1x TBE buffer for 15 min at 150 Volts, and amplified DNA visualised on a u.v. transilluminator.

2.4 RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS (RFLP)

Due to the high levels of sequence variation displayed by HCV, it is possible to distinguish between different genotypes and some subtypes, by the digestion of 5' NCR and core regions with specific restriction endonucleases (Mellor *et al.,* 1996; Davidson *et al.,* 1995; McOmish *et al.,* 1994).

Amplified 5'NCR was cleaved with 4 different restriction endonucleases (appendix 2.3), in two separate reactions; (i) *Rsa* I / *Hae* III and (ii) *Mva* I / *Hinf*

I (all Promega). Each double digest was incubated at 37°C overnight and contained 10 units (1μl) of each enzyme, 3 μl of the appropriate manufacturers 10x buffer and 25μl amplified PCR product. DNA fragments were then separated by electrophoresis through a 4% METAPHOR agarose (Flowgen) gel containing 0.05μg/ml ethidium bromide for 1 hour at 150 V. The combination of patterns from the two double digests was used to indicate the genotype of HCV present in the test sample (Smith *et al.,* 1995b; McOmish *et al.,* 1994b).

Subtypes of type 6 from South East Asia which produced RFLP patterns corresponding to type 1 with the above enzymes (Mellor *et al.,* 1996) were identified by an additional restriction digest using the enzymes *Dde* I / Hpa II.

DNA sequences of type 1 and novel variants of type 6 could be further distinguished by RFLP of the core region (Mellor *et al.,* 1996). Amplified core sequences were digested separately with the restriction endonucleases *Ava* I and *Sma* I. Type 6 subtypes were identified by the patterns a12s5, a10s1 or a13s5 (Mellor *et al.,* 1996). Reactions were carried out at 37°C overnight using 10 units restriction enzyme with 3μl manufacturers 10x buffer with 26μl amplified PCR product.

Subtypes a and b of genotypes 1, 2 and 3 were distinguished by RFLP of the 5' NCR using either *Bst*U1 (type 1) or *Scr*fI (types 2 and 3).

For type 1 samples, 25μl PCR product was digested with 5 units *Bst*U1, 0.3μl 1M $MgCl_2$, 0.375μl 4M NaCl, 0.3μl 0.1M DTT and 3.5μl distilled water at

60°C for a minimum of 4 hours. DNA fragments were separated by electrophoresis and subtypes identified according to the pattern of fragments produced (Davidson *et al.,* 1995). Subtypes 2a/b and 3a/b were identified by the digestion of 25μl PCR product with 5 units *Scr*fI in 3μl manufacturers 10x buffer and 1μl distilled water, at 37°C for a minimum of 4 hours.

2.5 DNA SEQUENCING

2.5.1 DIRECT SEQUENCING OF AMPLIFIED DNA PRODUCTS

Single stranded template DNA was prepared for sequencing using streptavidin coated magnetic beads (DYNABEADS; DYNAL Ltd.). Positive samples were re-amplified from the 1° PCR product in which one primer was biotinylated, in a final volume of 100μl. Beads (20μl per sample), were washed with 40μl 0.1% BSA pH 7.2, and then with 40μl BW (10mM tris HCl pH 7.5; 1mM EDTA; 2.0M NaCl). The beads were resuspended in 40μl BW, and incubated with an equal volume of PCR product for 20 minutes at room temperature. The efficient binding of biotinylated DNA to the streptavidin beads was promoted by gently resuspending the mixture several times during the incubation. The beads were washed in 40μl BW, and then resuspended in 8μl 0.15M NaOH for 10 min at room temperature to denature the DNA into single strands. The beads were then washed once in 50μl 0.15M NaOH, followed by 40μl BW and a final wash in 40μl TE buffer (10mM tris HCl; 1mM EDTA) pH 7.0, before being resuspended in 20μl TE.

Sequencing was performed using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemicals). Primer of opposite sense to the biotinylated primer was annealed to 5μl single stranded template DNA, by the addition of 2μl 5x annealing reaction buffer (200mM tris-HCl pH 7.5; 100mM

$MgCl_2$; 250mM NaCl), 2μl diluted primer (50ng) and 1μl DMSO (final concentration 10%). Tubes were heated to 65°C for 5 min, and then left to cool to room temperature over 20 minutes. Four termination mixes, ddGTP, ddATP, ddTTP and ddCTP were prepared (80μM each dNTP; 8μM specific ddNTP; 50mM NaCl; 10% DMSO) and 2μl aliquots of each incubated at 37°C. The labelling reaction was carried out by the addition of 1μl each of labelling mix (7.5μM dGTP, 7.5 μM dTTP, 1μM dCTP) diluted 1:20 in distilled water, 0.1M DTT, [α-^{35}S]-dATP and 2 units Sequenase (T7 DNA polymerase) diluted in TE pH 8.0, to the annealed DNA mixture, and incubating at room temperature for 2-5 min. Next, 3.5μl of labelling reaction was added to each of the termination solutions (G, A, T and C) and incubation was continued at 37°C for a further 5 min. The reactions were stopped by the addition of 4μl stop solution (95% formamide; 20mM EDTA; 0.05% bromophenol blue; 0.05% Xylene cyanol FF). DNA was denatured by heating at 90°C for 2 minutes and then separated by polyacrylamide gel electrophoresis (section 2.4.3).

2.5.2 SEQUENCING OF CLONED DNA FRAGMENTS

Double stranded plasmid DNA was denatured with alkali by adding 1μl 2M NaOH/2mM EDTA to 10μl miniprep DNA and incubated at 37°C for 30 minutes. The DNA was precipitated with 1μl 3M sodium acetate and 30μl ethanol at either -70°C for 15 minutes, or -20°C for more than 2 hours. A pellet of denatured DNA was obtained by centrifugation at 13000 revs / min for 10 minutes, and washed in

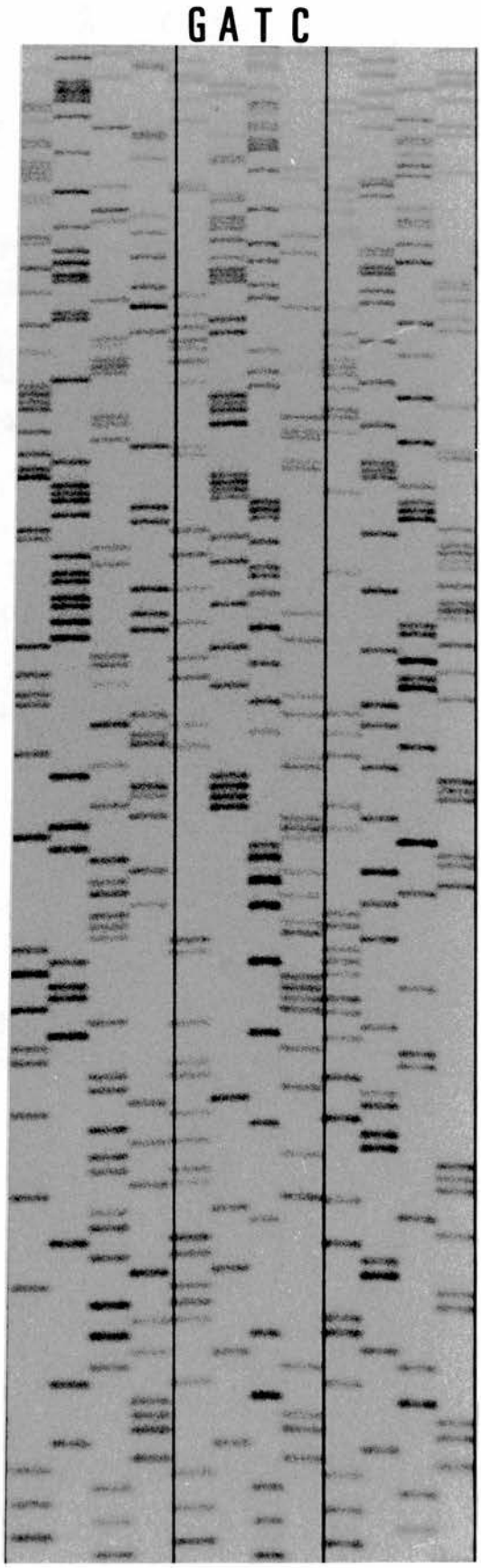
50μl 70% ethanol. The washed pellet was dissolved in 10μl DEPC water, and 5μl used for each sequencing reaction. DNA sequencing was carried out as in section 2.5.1, with plasmid primers 8819 (sense) and 9130 (antisense).

A typical sequencing gel is shown in figure 2.5.2.

2.5.3 DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

Denatured sequencing reactions were resolved by electrophoresis through a 6% polyacrylamide gel. Glass plates were thoroughly cleaned with distilled water, acetone and finally methanol, and assembled with 1mm wide spacers. Gels were prepared by dissolving 21g urea (AnalaR - BDH), 6ml Long Ranger Gel Solution (50% stock; Flowgen), 5ml 10x Sanger TBE (appendix 2.5) and 0.05g ammonium persulphate (APS) in distilled water to a final volume of 50ml. Polymerisation was initiated by the addition of 20μl TEMED (N, N, N', N'-Tetramethylethylediamine, SIGMA). after which the gel was poured immediately and left to set for approximately 1 hour. Denatured sequencing reactions were loaded onto the gel in the order G, A, T, C for each sample, and running buffer (1x Sanger TBE) added to fill both the anode and cathode chambers of the apparatus. Electrophoresis was carried out at 75 watts for 2-3 hours depending on the position of the sequence required in comparison with the primer used for sequencing. Gels were removed from the glass plates, transferred onto filter paper and vacuum dried at 80°C for 1.5 hours. The gels were then exposed to X-ray film (Kodak Biomax MR) in a light

Figure 2.5.2 A TYPICAL SEQUENCING GEL



tight cassette for 12-24 hours, and developed by kind permission of the radiography department at the Royal Infirmary of Edinburgh.

2.6 CLONING OF AMPLIFIED DNA INTO pTAg VECTOR

2.6.1 PREPARATION OF AMPLIFIED DNA FOR CLONING.

Amplified DNA fragments were cloned directly from PCR products into a dideoxy T (ddT) tailed vector (pTAg). This method (Marchuk *et al.,* 1991; Holton *et al.,* 1991) exploits the non-proofreading properties of *Taq* DNA polymerase (i.e. the absence of a 3' to 5' exonuclease activity which is present on certain other DNA polymerases) and its tendency to add a single adenosine (A) overhang to the 3' end of amplified PCR products.

The single A 3' overhangs are easily removed by nucleases, and so the highest efficiency of cloning is achieved by the use of unpurified PCR products, since there is a lower chance of nuclease contamination. To eliminate any residual polymerase activity, the PCR product was treated with an equal volume of chloroform:isoamyl alcohol (24:1), vortexed for 1 minute, and then spun in a microfuge (13000 rpm, 1 minute). The aqueous phase was then transferred to a new tube, and up to 2μl DNA used for ligation reactions. If the PCR reactions produced non-specific bands, the target DNA was purified using the Geneclean II kit (Bio 101 Ltd). The required DNA band was excised from an agarose-TBE gel with a scalpel, and transferred to a 1.5ml eppendorf. The total volume was made up to

100μl with DEPC water, before adding 3 volumes sodium iodide (NaI) stock solution and 50μl TBE modifier (if from an agarose / TBE gel) and melting the agarose in a water bath at 45-55°C for 5 minutes. 5μl resuspended glassmilk (silica matrix) was added to the DNA in solution and vortexed prior to centrifugation in a microfuge for 10 seconds. The pellet was then washed three times with 500μl NEW WASH (NaCl, ethanol, water), being careful to resuspend the pellet fully with each wash. DNA was finally eluted by resuspending the washed pellet in 5μl water and incubating at 45-55°C for 2 minutes. The glassmilk was pelleted by centrifugation for 30 seconds, and the supernatant containing DNA removed to a new tube. This elution step was repeated to give a final volume of 10μl. Prior to cloning, residual polymerase activity was eliminated as described above.

2.6.2 LIGATION OF DNA INSERT INTO pTAg

Amplified DNA fragment are ligated with plasmid vectors in a 1.5ml eppendorf, containing 50ng (1μl) pTAg vector, 1μl 10x ligase buffer (200mM Tris-HCl pH 7.6, 50mM $MgCl_2$), 0.5μl 100mM DTT and 0.5μl 10mM ATP. A maximum of 2μl PCR product was added to the tube and the volume made up to 9.5μl with nuclease free water. After mixing briefly on a vortex, the mixture was collected at the bottom of the tube by pulsing in a microfuge, and 0.5μl (2-3 Weiss units) T4 DNA ligase added. The solution was mixed gently with a pipette tip, and incubated at 16°C overnight.

2.6.3 TRANSFORMATION INTO E. COLI COMPETENT CELLS.

The introduction of plasmid DNA into E. coli cells (transformation) enabled the identification of copies which had been successfully ligated with the DNA insert. The efficiency of transformation is increased by the use of "competent" cells, which have been treated with calcium chloride to improve DNA binding to the cell wall, and therefore DNA uptake after heat-shock. The genotype of the competent cells provided with this kit was *end*A1 *hsd*R17(rk_{12}-m_{k13}+) *sup*E44*thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac*[F' *pro*A+B+*lac*I^qZ▲M15::Tn10(Tc^R)].

Competent cells were defrosted slowly on ice, and 20μl aliquots dispensed into pre-cooled eppendorf tubes. 1μl plasmid DNA from the ligation reaction was added to the cells and mixed by tapping gently. After a 30 minute incubation on ice, the tubes were heat-shocked at 42°C for 40 seconds and returned to ice for 2 minutes. 80μl SOC medium was added to the cells, which were then grown at 37°C for 1 hour in a rotary shaking incubator (200-250rpm). The cells were spread on selective LB agar plates, containing 50μg/ml ampicillin, 15μg/ml tetracycline, 80μg/ml X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-thiogalactoside) to 0.5mM. The presence of ampicillin ensures the presence of only transformants with the plasmid which carries the gene for ampicillin resistance (β-lactamase), and tetracycline the presence of the Lac Z gene on the F' plasmid of the cell. Transformants containing the DNA insert were identified by screening for β-galactosidase activity. pTAg contains the LacZα peptide sequence, which acts together with the LacZ peptide synthesised in

competent cells to produce the enzyme β-galactosidase. This enzyme, when in the presence of an inducer such as IPTG, is able to degrade the lactose analogue X-gal into a blue product. When a DNA insert is cloned into the plasmid, the LacZ gene is interrupted and therefore usually not expressed in an active form. The result is an absence of β-galactosidase activity, leaving X-gal intact and the colony white.

2.6.4 SCREENING OF COLONIES FOR DNA INSERTS

White colonies were screened for the presence of a DNA insert by PCR (Gussow *et al.,* 1990). Using a sterile toothpick, the edge of the colony was transferred into a tube containing 20μl PCR reaction mix (section 2.3.3) containing plasmid primers 8819 (sense) and 9130 (antisense) (table 2.3). The solution was overlayed with paraffin oil and placed on a thermal cycler for 30 cycles of 94°C for 1.5 minutes, 50°C for 30 seconds and 72°C for 3 minutes, followed by an incubation at 20°C for 7 minutes. PCR products were electrophoresed through a 2% agarose/TBE gel containing 0.05μg/ml ethidium bromide for 15 minutes with molecular weight markers for reference. Colonies giving rise to amplified DNA fragments of the correct size were selected.

2.6.5 PREPARATION OF PLASMID DNA

E. coli colonies containing DNA inserts were picked and grown in 3ml LB (appendix 2.6) at 37°C overnight in a rotary shaking incubator (200-250 rpm). The culture was split, with 1.5ml added to 0.5ml sterile glycerol and stored at -20°C,

and the other 1.5ml used for the preparation of plasmid miniprep DNA by alkaline denaturation.

In a 1.5ml Eppendorf tube, the culture was centrifuged in a microfuge for 15 seconds to pellet the cells, and the supernatant discarded. Bacterial cell wall polymers were degraded by resuspending the pellet in 100μl GTE (50mM glucose; 25mM Tris.Cl pH 8.0; 10mM EDTA) with lysozyme (SIGMA). The pH of the solution was raised to 12.5 by the addition of 200μl NaOH/SDS (ice, 5 minutes) to denature the DNA (plasmid DNA can renature as it is supercoiled), followed by the addition of acid (solution III; appendix 2.6) to aggregate the denatured DNA, making it insoluble. The tubes were centrifuged in a microfuge for 8 minutes to pellet the cellular DNA and debris, and the supernatant containing plasmid DNA transferred to a new tube. Plasmid DNA was precipitated by adding 290μl isopropanol, incubating at room temperature for at least 15 minutes, and pelleting in a microfuge at 13000 revs / min for 10 minutes. The pellet was washed with 500μl 70% ethanol prior to resuspending in 40μl TE containing RNase to a final concentration of 20μg/ml.

2.7 PROTEIN EXPRESSION

2.7.1 EXPRESSION VECTOR

Cloned DNA sequences were expressed using the pRSET "Xpress System"™ (Invitrogen corporation) (Kroll *et al.,* 1993). This system is an adaptation of that devised by Studier *et al* (1990) (Studier *et al.,* 1990) whereby protein expression is driven by T7 RNA polymerase acting on the T7 promoter present upstream of the pRSET multiple cloning site (figure 2.7.1). Also upstream of the DNA insert is a fusion peptide, whose sequence encodes an ATG translation initiation codon, a row of six histidine residues, a transcript stabilizing sequence derived from gene 10 of bacteriophage T7 and an enterokinase cleavage site. The hexahistidine tag is present to provide a metal binding domain, which allows rapid purification of the expressed protein by Metal Chelate Affinity Chromatography (MCAC) on nickel ion affinity resins.

The vector is transformed into *E.coli* strain JM109, and protein expression induced via derepression of the *lac* promoter (by the addition of IPTG), which subsequently induces the expression of T7 RNA polymerase from M13/T7 bacteriophage when added to the culture. It is this enzyme which finally acts on the T7 promoter in the vector to drive expression of the cloned DNA sequence.

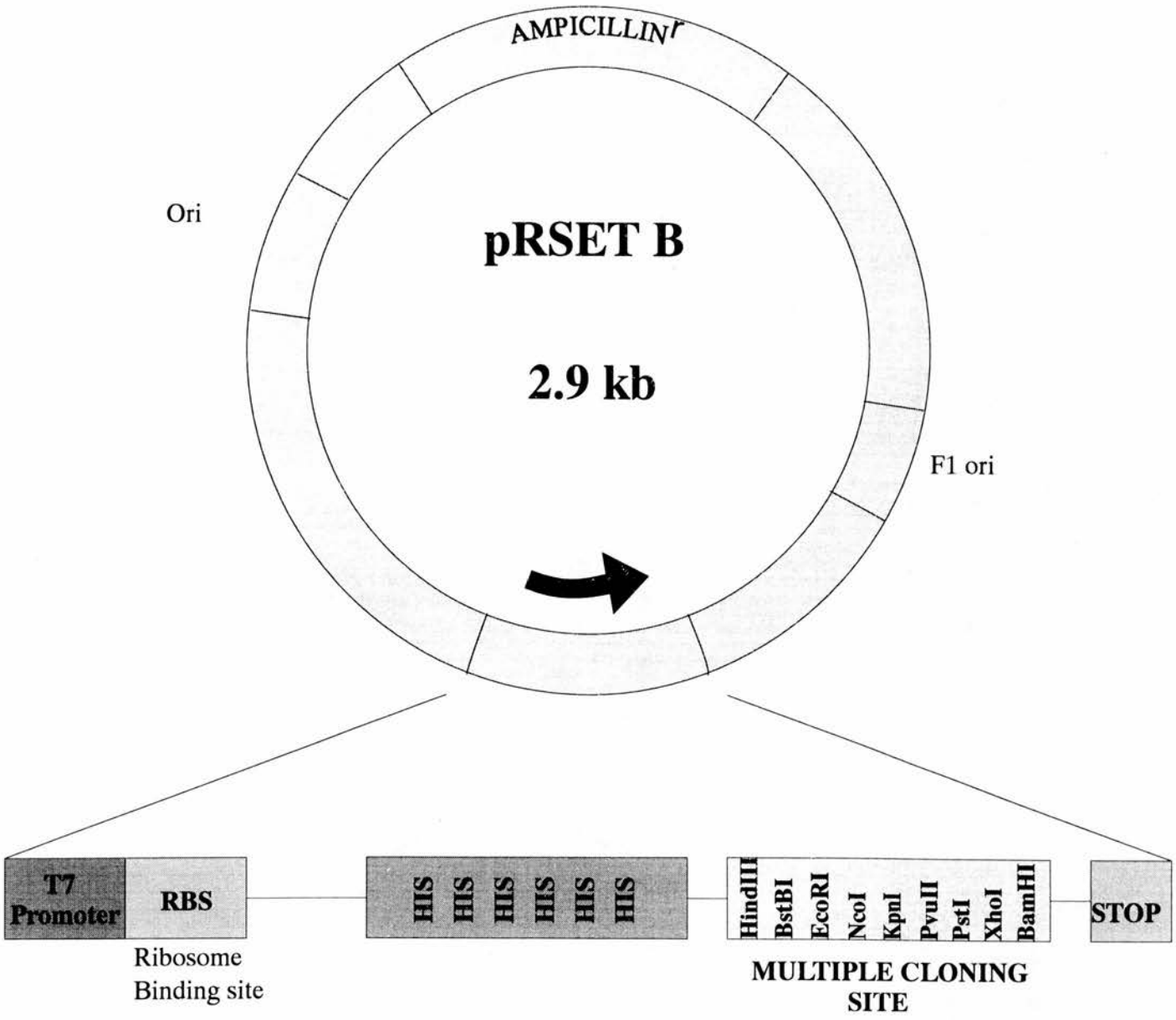
Figure 2.7.1 VECTOR MAP OF pRSET B

A vector map of pRSET B, showing the origin of replication (ori) and the F1 origin (F1 ori), with the hexahistidine tag located upstream of the multiple cloning site.

HIS; histidine codon

STOP; termination codon

Amplicillin^r; Ampicillin resistance gene (β-Lactamase)


AMPICILLIN^r
Ori
pRSET B
2.9 kb
F1 ori
T7 Promoter
RBS
Ribosome Binding site
HIS HIS HIS HIS HIS HIS
HindIII BstBI EcoRI NcoI KpnI PvuII PstI XhoI BamHI
MULTIPLE CLONING SITE
STOP

2.7.2 SUBCLONING INTO pRSET

DNA sequences cloned into pTAg were subcloned into pRSET for protein expression. The DNA insert was isolated from pTAg by restriction endonuclease digestion with the enzymes BamHI / HindIII, by the addition of 0.5μl (5 units) each enzyme and 2μl 10x Buffer E (Promega) to 17μl miniprep DNA followed by incubation at 37°C for 1 hour. Products were electrophoresed through a 1% TAE agarose gel containing 0.05μg/ml ethidium bromide. Along with molecular weight markers bands of the appropriate size were excised from the gel and purified as described previously (section 2.6.1). DNA fragments were then ligated with pRSET (previously cleaved with BamHI / HindIII) by the addition of 5μl miniprep DNA to 3μl cleaved pRSET, 2μl 10x Ligase Buffer, 0.5μl T4 DNA ligase and nuclease free water to a final volume of 20μl. Reactions were incubated at room temperature overnight prior to transformation into JM109 *E.coli* competent cells (kindly provided by Murex Biotech, genotype *rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi*Δ(*lac-pro*AB)F'[*tra*D36 *pro*AB+ *lac*I^q *lac*ZΔM15]) as described previously with the exception that 50μl cells per reaction were used with 5μl ligated DNA, and transformed cells were grown in 450μl SOC medium. Transformants were selected by plating on SOB agar (appendix 2.7) which contained ampicillin at 50μg/ml. Colonies were screened for the presence of the DNA insert by direct PCR using pRSET primers 632 and 662 (Murex Biotech, table 2.3) as previously described (section 2.6.4), and positive colonies grown overnight in 10ml SOB broth containing 50μg/ml ampicillin.

2.7.3 PILOT EXPRESSION

Cloned DNA sequences were initially expressed in 50ml cultures, to determine the kinetics of the reaction and the conditions required for maximum yield of expressed protein. 5ml *E.coli* overnight culture was transferred to a sterile 250ml conical flask containing 45ml SOB broth with ampicillin (50μg/ml), and incubated at 37°C in a rotating incubator. When the $O.D._{600}$ of the culture reached 0.3, IPTG was added to a final concentration of 1mM, and growth continued at 37°C for 1 hour. The $O.D._{600}$ of the culture was measured again, and the cell concentration calculated on the basis that an $O.D._{600}$ of 1.0 represents a concentration of 10^9 cells/ml. Protein expression was induced by the addition of M13/T7 bacteriophage (kindly provided and titred by Murex Biotech) to the culture at a multiplicity of infection (MOI) of 5 plaque forming units (pfu) per cell, and the new cultures incubated overnight at 37°C in a rotating incubator. The timecourse of protein production was monitored by removing 1ml samples from the cultures, pelleting the cells in a microfuge, and resuspending them in 100μl distilled water before analysis by SDS-PAGE (section 2.7.4) and Western blotting (section 2.7.5).

At the end of the culture period (overnight), the cells were pelleted in a centrifuge at 5000 rpm for 10 minutes, the supernatant aspirated and the pellet resuspended in 1ml Lysis buffer (appendix 2.7). *E.coli* cell walls were degraded by adding 10μl lysozyme (10mg/ml) to the sample and incubating at room temperature for 1 hour (swirling continuously). 10μl sodium deoxycholate (5% w/v) was added

to the tubes to solubilise the sarcoplasmic reticulum, at which stage the solution becomes viscous, and finally 10μl 1M Mg SO_4 and 4μl (40-200 Units) DNase (RNase free; Boehringer). The tubes were incubated at room temperature with swirling for 1 hour, or until the viscosity disappeared. Inclusion bodies and other insoluble material were pelleted in a microfuge at 13000 rpm for 5 minutes, the supernatant transferred to a new tube, and the pellet resuspended in 1ml 8M urea containing 10mM DTT. Proteins expressed from cloned DNA were present in either the periplasm of the *E.coli* cells (supernatant) or in inclusion bodies (cell pellet), and were identified by SDS-PAGE.

2.7.4 DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

Proteins were separated according to their molecular weight by denaturing polyacrylamide gel electrophoresis (PAGE). Proteins were denatured in the presence of SDS, an anionic detergent which disrupts noncovalent interactions between and within proteins, and DTT which disrupts disulphide bonds. SDS anions bind to the denatured protein to give a net charge proportional to that of the mass, and proteins are then separated by electrophoresis towards the anode.

Two glass plates were cleaned first with water, and then methanol, and assembled with 0.75mm spacers in the manufacturers stand (BioRad). A 15% resolution gel was prepared (appendix 2.7), and poured between the plates using a pipette until the level reached approximately two thirds from the bottom. The

surface of the gel was overlayed with methanol, and left to polymerise at room temperature for 45 minutes. The methanol was poured off, and a 15% stacking gel prepared (appendix 2.7). Stacking gel solution was poured on top of the resolution gel, and a 0.75mm comb placed between the plates. This was again left to polymerise at room temperature for 45 minutes. The comb was removed carefully, and the gel placed into the electrophoresis apparatus (BioRad), and submerged with 1x SDS-PAGE running buffer (appendix 2.7).

Samples were prepared by the addition of an equal volume of 2x SDS sample buffer (appendix 2.7), and heating to 100°C for 5 minutes. Samples were then loaded into the appropriate wells using a flat tipped pipette and electrophoresed at 200 Volts for 45 minutes. Protein bands were visualised by soaking the gel in Coomassie blue staining solution (appendix 2.7) for 30 minutes, followed by the removal of residual dye with destaining solution (appendix 2.7) for 2 hours - overnight.

2.7.5 WESTERN BLOTTING

Expressed proteins were distinguished from *E.coli* proteins by Western blotting (Salinovich *et al.*, 1986) of polyacrylamide gels onto nitrocellulose membranes, and probing with antibody specific for the hexahistidine tag. The gels were transferred to nitrocellulose membranes which had been activated by soaking in methanol. Sandwiched between 4 pieces of filter paper soaked in 1x transfer buffer (appendix 2.7), the gel and membrane were placed on electrophoresis

apparatus (Biorad) with the nitrocellulose membrane facing the anode, and a current of 350 mA applied for 45 minutes. The membrane was removed and soaked in 1x cast solution (appendix 2.7) for 1 hour at room temperature, and then reacted with conjugate CM-POD (Sigma) at a concentration of 1:1000 (1mg/ml stock) in 1x cast solution for 30 minutes. After washing 3 times in PBS/Tween, the colour was developed with diaminobenzidine (DAB) substrate for 2-3 minutes, and the reaction stopped by washing in water.

2.7.6 LARGER-SCALE PROTEIN PREPARATION

To prepare quantities of antigen sufficient for ELISA work, expression of cloned DNA was carried out in 2 X 500ml cultures per sample. The procedure was as for pilot expression except that lysozyme was added to a final concentration of 4μg/ml for 30 minutes at 4°C followed immediately by sonication of the cells on ice for 3 x 30 seconds with 15 second intervals, and prior to the separation of insoluble material by centrifugation, 5ml 10% Triton buffer TX 100 was added to the solution.

Purification of Expressed proteins by Metal Chelate Affinity Chromatography

Proteins expressed in a vector with an N-terminal fusion peptide of polyhistidine residues were purified by Metal Chelate Affinity Chromatography (MCAC) (Hochuli, 1990; Porath *et al.*, 1975) on columns containing ProBond™ Resin (Invitrogen corporation). The resin, consisting of nickel ions (Ni^{2+}) covalently

bound to nitrilotriacetic acid (NTA) (Hochuli *et al.,* 1987), binds to the hexahistidine tail of the expressed protein via the imidazole side chains of the histidine residues, leaving *E.coli* proteins to be washed through the column. Bound protein is eluted from the resin by washing with a high concentration of imidazole, which at a constant pH will displace the polyhistidine tail from the Ni^{2+}. All buffers used in this process are described in appendix 2.9.

ProBond™ columns were filled with resuspended resin until the packed volume reached the top of the narrow section of the column, and equilibrated with 10ml 1x Binding buffer. Prepared cell lysate (containing soluble expressed protein) was loaded onto the column, and the flowthrough collected for analysis by SDS-PAGE. The resin was washed three times with 10ml 1x Binding buffer, followed by individual washes with 10mM imidazole solution and 30mM imidazole solution. Histidine-tagged proteins were finally eluted with ten 1ml washes of 300mM imidazole solution. Fractions containing the purified protein were identified by SDS-PAGE of the flowthrough samples.

CHAPTER 3

3 DEVELOPMENT AND APPLICATION OF A SEROTYPING ASSAY FOR HCV TYPES 1-6.

3.1 HCV 1-3 ASSAY

3.1.1 INTRODUCTION

The hypothesis that amino acid variation between HCV genotypes could generate a type-specific immune response towards certain epitopes was put forward shortly after the introduction of the first-generation enzyme immunoassays for the routine serological screening of blood donors. It was observed that serological reactivity towards NS4 antigens specific for type 1 virus (for example c100-3 and 5-1-1) was relatively infrequent amongst sera from individuals infected with types 2 or 3 (McOmish *et al.,* 1993). As sequences of genotypes 1 and 2 encoding the c100-3 antigen show only 75-77% amino acid identity, the existence of type specific antibodies to this antigen was conceivable.

3.1.2 DEVELOPMENT OF A SEROTYPING ASSAY FOR HCV TYPES 1-3.

Consensus sequences for genotypes 1, 2 and 3 were compared and a set of 82 oligopeptides spanning NS4 (amino acid residues 1679-1768) prepared for each. Each peptide was 9 residues in length and overlapped the adjacent peptide by 8 residues. The antibody reactivity towards individual peptides was confined to three specific regions, of which two were at the same positions for all genotypes.

Antigenic region 1 included amino acid residues 1691-1708, and region 2 spanned positions 1720-1738. The different positions within NS4 of antigenic region 1 (NS4A) and region 2 (NS4B) are such that they are cleaved *in vivo*. The level of cross-reactivity between antibody and peptides from different genotypes was investigated by the reaction of sera from HCV infected blood donors with heterologous peptides, and by comparing results with the reactivity against peptides from a homologous genotype to that causing infection. Although cross-reactivity was observed, this was weak and generally confined to region 1, with the exception of type 3 antibody, which showed weak cross-reactivity with type 1 peptides in both regions 1 and 2.

The discovery of type-specific antibody towards epitopes in the NS4 region led to the development of a serotyping ELISA for genotypes 1, 2 and 3 (Simmonds *et al.,* 1993d). Branched peptides representing antigenic regions 1 and 2 of genotypes 1a/b, 2b and 3a were synthesised on *p*-hydroxymethylphenoxymethylpolystyrene resin with a branched lysine core, to obtain eight copies of the sequence per molecule and the maximum level of binding. Variations between single amino acid residues within type 1 were accommodated by the incorporation of equimolar amounts of the alternatives, resulting in a total of 8 peptides, which were coated onto microtitre plates at equal concentrations.

In this assay, reactivity of sera from HCV infected blood donors to type-homologous peptides was observed more frequently than towards type-heterologous antigen. In order to eliminate the cross-reactivity observed between different genotypes, the assay was modified by the addition of a one hundred fold excess concentration of type-heterologous peptides. All 8 peptides were present on the solid phase, and blocking solutions of heterologous peptides were added to three separate typing wells (i.e. the well responsible for the identification of type 1 specific antibody would have a blocking solution containing type 2 and 3 peptides added). No competing peptides were added to a positive control well, while all 8 peptides were added at blocking concentrations to a negative control well. Initial studies revealed that this assay was able to detect NS4 antibody in 95%, and type-specific antibody in 89% HCV positive blood donors from Scotland. Serotyping results were concordant with the genotype identified by RFLP of the 5'NCR in 99.2% samples (Simmonds *et al.,* 1993d).

3.1.3 RESULTS

Application of the 1-3 Serotyping Assay to Samples from Patients in the U.S.A.

The 1-3 serotyping assay was used for the detection of type-specific antibody in a total of 217 samples obtained from patients with chronic hepatitis C in the United States. Genotyping of the samples by RFLP analysis of the 5' NCR revealed that there were 163 type 1, 30 type 2 and 11 type 3 infections, while 13 sera were negative by PCR. The serotyping assay detected type-specific antibody

Table 3.1.3 DETECTION OF TYPE-SPECIFIC ANTIBODY IN SAMPLES FROM PATIENTS WITH CHRONIC HEPATITIS C IN THE U.S.A. USING THE 1-3 SEROTYPING ASSAY

Genotype (RFLP)	Serotype 1	2	3	NTS*	NR†	Total	Sensitivity (%)	Concordance (%)
1	148	-	-	11	4	163	90.8	100
2	1	28	-	-	1	30	96.7	96.6
3	1	-	8	1	1	11	81.8	88.9
PCR -ve	9	2	-	1	1	13	84.6	n/a ‡
All samples	159	30	8	13	7	217	90.8	98.9 ¶

* Non-type-specific reactivity (appendix 1.0)
† Non reactive
‡ Not applicable as genotyping was not possible for these samples
¶ Calculation based on the results from samples which could be genotyped by RFLP

in 148/163 (90.8%) type 1 samples with 100% specificity (Table 3.1.3). The sensitivity of the assay towards samples infected with type 2 virus was higher at 96.7%, but in one sample type 1 specific antibody was detected. Similarly, one sample from an individual infected with genotype 3 virus was type 1 by serotyping. The majority (11/13) of PCR negative samples could be serotyped, revealing nine serotype 1 samples and two serotype 2 samples. Overall, the sensitivity of the 1-3 serotyping assay in this study was 91.2%, with type-specific antibody detected corresponding to the genotype identified by RFLP in 98.9% samples.

3.1.4 DISCUSSION

Shortly after the development of the serotyping ELISA for HCV types 1-3, the analysis of sera from a broader range of geographical areas led to the discovery of additional genotypes of HCV. Type 4 was discovered initially in Africa, type 5 in South Africa and type 6 in Hong Kong (reviewed in Simmonds, 1993a). The sensitivity of the serotyping assay was greatly reduced in populations where genotypes 4, 5 and 6 were prevalent, as antigens for these additional genotypes were not present.

In addition to the discovery of genotypes 4-6, a more intensive investigation of the genotype distribution within European countries revealed that subtypes 2a and possibly 2c were as common as type 2b (Davidson *et al.,* 1995), and that the use of type 2b peptides alone may reduce the sensitivity of the assay to these subtypes (discussed in chapter 4).

3.2 DEVELOPMENT OF A SEROTYPING ASSAY FOR THE DETECTION OF HCV GENOTYPES 1-6.

3.2.1 INTRODUCTION.

In order to be able to design type-specific NS4 peptides for genotypes 4, 5 and 6 it was necessary to compare a number of sequences for each. In total, six type 4 samples, four type 5 samples and two type 6 samples were obtained from HCV positive blood donors in Egypt, South Africa and Hong Kong respectively (Bhattacherjee *et al.,* 1995).Virus genotypes were confirmed by comparison of 5' NCR sequences with published sequences.

For each sample, a portion of the NS4 region including both antigenic regions 1 and 2 was amplified and sequenced (Figure 3.2.1). The inferred amino acid sequences were used to produce a consensus sequence for each genotype from which type-specific antigenic region 1 and 2 peptides could be designed (Table 3.2.1). A total of 21 branched peptides corresponding to the consensus sequences for all six genotypes were synthesised.

PCR amplification and sequence analysis of samples containing types 4, 5 and 6 was carried out by Vasker Bhattacherjee, while the branched peptides were synthesised by Dr. Brian Rodgers at Murex Biotech, Dartford, U.K.

Figure 3.2.1 A COMPARISON OF NS4 AMINO ACID SEQUENCES FROM DIFFERENT GENOTYPES

HCV-PT HCV prototype sequence; Choo *et al.*, 1991

"." Sequence identity to HCV-PT

"?" sequence not determined

Antigenic regions 1 and 2 are highlighted in bold

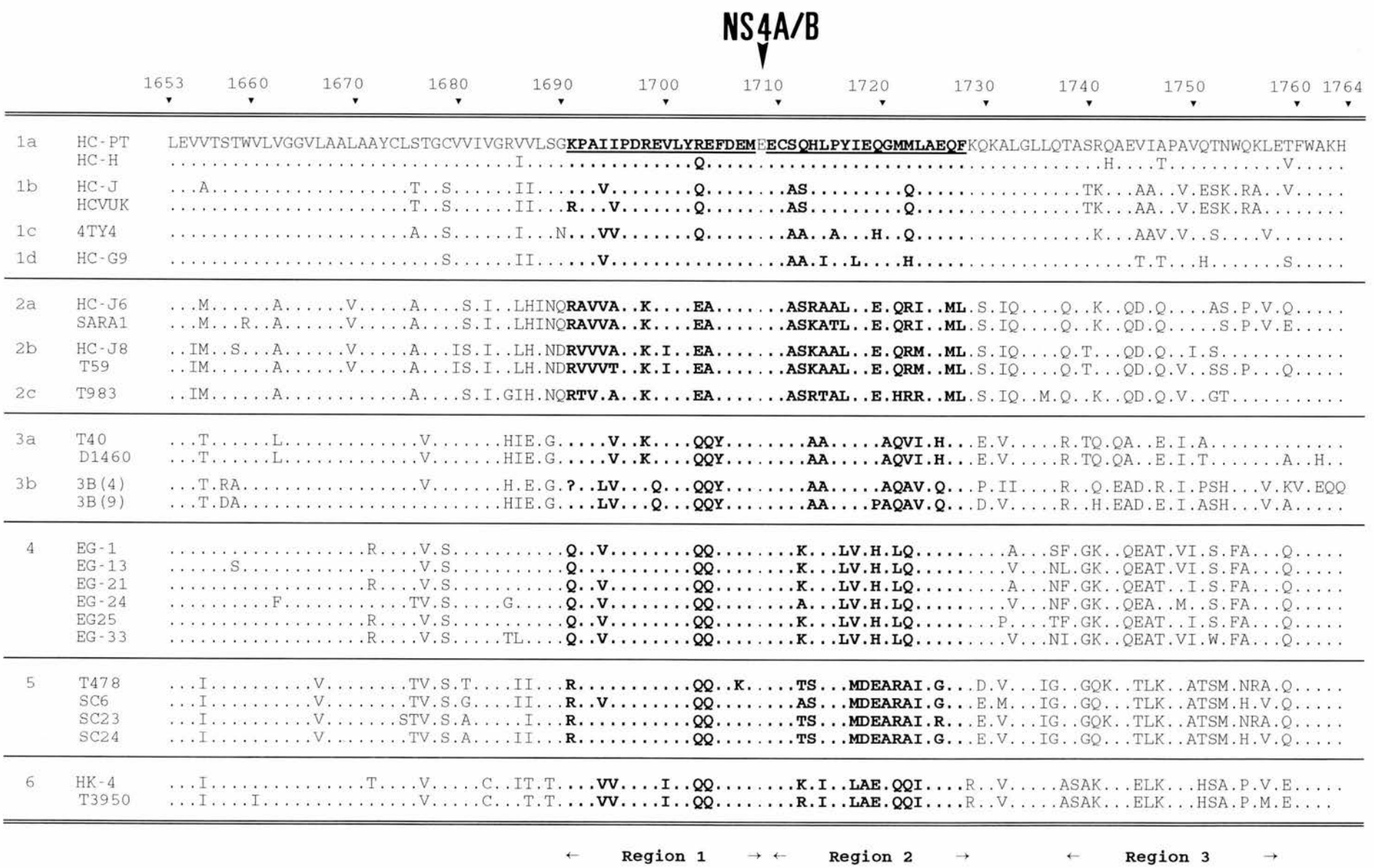

NS4A/B
1653
1660
1670
1680
1690
1700
1710
1720
1730
1740
1750
1760
1764
1a HC-PT LEVVTSTWVLVGGVLAALAAYCLSTGCVVIVGRVVLSGKPAIIPDREVLYREFDEMEECSQHLPYIEQGMMLAEQFKQKALGLLQTASRQAEVIAPAVQTNWQKLETFWAKH
HC-H .....I.....Q.....H....T.....V.....
1b HC-J ...A.....T..S.....II.....V.....Q.....AS.....Q.....TK...AA..V.ESK.RA..V.....
HCVUK .....T..S.....II...R...V.....Q.....AS.....Q.....TK...AA..V.ESK.RA.....
1c 4TY4 .....A..S.....I...N...VV.....Q.....AA..A...H..Q.....K...AAV.V..S....V.....
1d HC-G9 .....S.....II.....V.....AA.I..L....H.....T.T...H.....S.....
2a HC-J6 ...M.....A.....V.....A....S.I..LHINQRAVVA..K....EA.....ASRAAL..E.QRI..ML.S.IQ....Q..K..QD.Q....AS.P.V.Q.....
SARA1 ...M...R..A.....V.....A....S.I..LHINQRAVVA..K....EA.....ASKATL..E.QRI..ML.S.IQ....Q..K..QD.Q.....S.P.V.E.....
2b HC-J8 ..IM..S...A.....V.....A...IS.I..LH.NDRVVVA..K.I..EA.....ASKAAL..E.QRM..ML.S.IQ....Q.T...QD.Q..I.S.....
T59 ..IM.....A.....V.....A...IS.I..LH.NDRVVVT..K.I..EA.....ASKAAL..E.QRM..ML.S.IQ....Q.T...QD.Q.V..SS.P...Q.....
2c T983 ..IM.....A.....A....S.I.GIH.NQRTV.A..K....EA.....ASRTAL..E.HRR..ML.S.IQ..M.Q..K..QD.Q.V..GT.....
3a T40 ...T.....L.....V.....HIE.G.....V..K....QQY.....AA.....AQVI.H...E.V.....R.TQ.QA..E.I.A.....
D1460 ...T.....L.....V.....HIE.G.....V..K....QQY.....AA.....AQVI.H...E.V.....R.TQ.QA..E.I.T.....A..H..
3b 3B(4) ...T.RA.....V.....H.E.G.?..LV...Q...QQY.....AA.....AQAV.Q...P.II....R..Q.EAD.R.I.PSH...V.KV.EQQ
3B(9) ...T.DA.....HIE.G....LV...Q...QQY.....AA....PAQAV.Q...D.V.....R..H.EAD.E.I.ASH...V.A.....
4 EG-1 .....R....V.S.....Q..V.....QQ.....K...LV.H.LQ.....A...SF.GK..QEAT.VI.S.FA...Q.....
EG-13 .....S.....V.S.....Q.....QQ.....K...LV.H.LQ.....V...NL.GK..QEAT.VI.S.FA...Q.....
EG-21 .....R....V.S.....Q..V.....QQ.....K...LV.H.LQ.....A...NF.GK..QEAT..I.S.FA...Q.....
EG-24 .....F.....TV.S.....G.....Q..V.....QQ.....A...LV.H.LQ.....V...NF.GK..QEA..M..S.FA...Q.....
EG25 .....R....V.S.....Q..V.....QQ.....K...LV.H.LQ.....P....TF.GK..QEAT..I.S.FA...Q.....
EG-33 .....R....V.S.....TL....Q..V.....QQ.....K...LV.H.LQ.....V...NI.GK..QEAT.VI.W.FA...Q.....
5 T478 ...I.....V.....TV.S.T....II...R.....QQ..K.....TS...MDEARAI.G...D.V...IG..GQK..TLK..ATSM.NRA.Q.....
SC6 ...I.....V.....TV.S.G....II...R..V.....QQ.....AS...MDEARAI.G...E.M...IG..GQ...TLK..ATSM.H.V.Q.....
SC23 ...I.....V.....STV.S.A.....I...R.....QQ.....TS...MDEARAI.R...E.V...IG..GQK..TLK..ATSM.NRA.Q.....
SC24 ...I.....V.....TV.S.A....II...R.....QQ.....TS...MDEARAI.G...E.V...IG..GQ...TLK..ATSM.H.V.Q.....
6 HK-4 ...I.....T....V.....C..IT.T....VV....I..QQ.....K.I..LAE.QQI....R..V.....ASAK...ELK...HSA.P.V.E.....
T3950 ...I....I.....V.....C...T.T....VV....I..QQ.....R.I..LAE.QQI....R..V.....ASAK...ELK...HSA.P.M.E....
← Region 1 → ← Region 2 → ← Region 3 →

Table 3.2.1 **NS4 peptides used in the 1-6 Serotyping ELISA.**

HCV TYPE	ANTIGENIC REGION 1	ANTIGENIC REGION 2
1a	KPAIVPDREVLYREFDEM	ECSQHLPYIEQGMMLAEQF
1b	R..VI.....Q.....	.....A.....
2a	RAV.A..K....EA....	..ASRAAL..E.QRI..ML
2b	RVVVT..K.I..EA....	..ASRAAL..E.QRI..ML
2c	RTV.A..K....EA....	..ASRTAL..E.HRI..ML
3	...L...K....QQY...	....AA.....AQVI.H..
4	Q..VI.....QQ....	...K...LV.H.LQ.....
5	R...I.....QQ....	...TS...MDEARAI.G..
6	...VV....I..QQ....	...R.I..LAE.QQI....

Table reproduced from Bhattacherjee *et al.* 1995.

"." Sequence identity to the type 1a sequence

3.2.2 ASSAY FORMAT

The overall design of the assay was the same as for the 1-3 serotyping ELISA but with a few minor modifications. As the total number of peptides had now been increased to 21 and the number of peptides representing each of the six genotypes was not the same, the concentrations used for coating plates was adapted. Equal concentrations of each individual peptide were used to coat plates for the 1-3 serotyping ELISA, whereas the final concentrations of peptides specific to each genotype were equal for coating plates for the 1-6 assay. Concentrations of blocking peptides were a 100 fold excess of that used for coating the plates as before. Better results were obtained with a 1:20 test dilution of serum rather than 1:40 used for the 1-3 assay, and finally the layout of the plate was modified in order to fit the maximum number of samples per plate, with one column of 8 wells per sample (Figure 2.2.4).

3.2.3 SAMPLES.

The different genotypes of HCV show specific geographical distributions, with types 1-3 existing in a wide range of areas compared with types 4, 5 and 6 which exist in relatively confined regions (Central Africa and the Middle East, South Africa and South East Asia respectively). A thorough evaluation of the capability of the 1-6 ELISA to identify type-specific antibody for these types was carried out.

All the samples used in this investigation were analysed by RFLP of amplified 5'NCR sequences in order to identify the genotype of HCV causing infection. Type 1 samples (n=39) were obtained from Norwegian patients with chronic HCV (section 3.3), and included subtypes 1a (n=25) and 1b (n=12). Samples from individuals infected with type 2a (n=14) were acquired from HCV positive blood donors in Taiwan (section 3.3), whereas samples infected with type 2b (n=18) were mostly from the Norwegian patients, with only two samples from Taiwan. Samples infected with type 3 (n=55) were also from Norway. Types 4 (n=62), 5 (n=16) and 6 (n=6) were obtained from HCV positive blood donors in Egypt, South Africa and Hong Kong respectively. A total of 210 HCV positive samples were analysed using the 1-6 assay, along with 20 samples from healthy blood donors as negative controls (Table 3.2.4).

3.2.4 RESULTS.

From 210 HCV positive samples tested with the assay, type-specific antibody was detected in a total of 183, giving an overall sensitivity (Appendix 1.0) of 87.1%. The type-specific antibody detected in 178 of these samples was in agreement with genotyping by RFLP, giving the ELISA an overall specificity of 97.3% (Table 3.2.4).

Although the specificity (appendix 1.0) was high for the majority of genotypes analysed, the sensitivity of the assay for different genotypes was more variable. From 39 type 1 sera tested, type-specific antibody was detected in 36

Table 3.2.4 DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV POSITIVE SERA OF KNOWN GENOTYPE.

Genotype (RFLP)		Serotype 1	2	3	4	5	6	NTS*	NR†	Total	Sensitivity‡ (%)	Concordance‡ (%)
1	All	35	-	-	-	-	1	1	2	39	92.3	97.2
	a	24	-	-	-	-	-	-	1	25	96.0	100
	b	9	-	-	-	-	1	1	1	12	83.3	90.0
2	All	1	30	-	-	-	-	-	1	32	96.9	96.8
	a	-	13	-	-	-	-	-	1	14	92.9	100
	b	1	17	-	-	-	-	-	-	18	100	94.4
3		-	1	50	-	-	-	2	2	55	92.7	98.0
4		-	1	-	49	-	-	9	3	62	80.6	98.0
5		1	-	-	-	10	-	2	3	16	68.8	90.9
6		-	-	-	-	-	4	1	1	6	66.6	100
Total		37	32	50	49	10	5	15	12	210	87.1	97.3

* Non-type-specific reactivity
† Non reactive
‡ Terms explained and calculated as described in appendix 1.0.

(sensitivity 92.3%) with only one discrepant result (genotype 1b, serotype 6). However, for subtype 1a both the sensitivity (96%) and specificity (100%) of the assay were higher than for subtype 1b (83.3% and 90%). High performance criteria were observed for the 32 samples from individuals infected with type 2 with only 1 sample showing no reactivity (NR) to the NS4 peptides, and 1 giving a discrepant result being identified as serotype 1. Type-specific antibody was detected in 51/55 type 3 samples (sensitivity 92.7%) with 1 discrepancy (identified as serotype 2). The sensitivity of the ELISA towards type 4 was lower at 80.6%, with 9 samples untypeable by the assay despite showing reactivity towards the NS4 peptides in the positive control well (NTS; non-type-specific), and 3 samples in which no NS4 antibody could be detected. The sensitivity of the assay to types 5 and 6 samples were also lower, although fewer samples were available for analysis. Type-specific antibody was detected in 11/16 type 5 samples, with 1 discrepant result (serotype 1), and similarly only 4/6 type 6 samples could be serotyped by the assay. All the negative controls were unreactive.

3.2.5 DISCUSSION

The production of type-specific antibody directed towards epitopes of HCV which show relatively high levels of amino acid variation between genotypes has been exploited by a number of workers for the development of serological typing assays (Tanaka *et al.*, 1994; Machida *et al.*, 1992). However, these assays were restricted to the detection of types 1 and 2, and as the identification of serotype

was based on a direct comparison of reactivity towards different antigens, they carried a potential risk of cross-reactivity and hence the misidentification of serotypes. The development of a competitive ELISA for the identification of types 1-3 (Simmonds *et al.,* 1993d) largely eliminated this risk and was demonstrated as being both sensitive and accurate within populations containing these genotypes, although the subsequent discovery of additional genotypes demanded an assay with greater capabilities for use worldwide.

Analysis of amplified NS4 sequences for genotypes 4, 5 and 6 revealed type-specific differences in the two antigenic regions previously identified by epitope mapping (Simmonds *et al.,* 1993d), although types 4-6 were relatively less distinct from type 1 in antigenic region 1. Peptides were synthesised towards consensus sequences for types 4-6, and incorporated into the serotyping assay.

From a total of 210 PCR positive samples from individuals infected with a full range of HCV genotypes, the work described here has shown that the 1-6 serotyping assay was able to identify type-specific antibody in 183 (87.1%) (Table 3.2.4). All these samples were HCV positive by PCR. The samples showing no reactivity towards the NS4 antigens (NR) could in theory have been obtained from immunocompromised patients, for example, those who were co-infected with HIV or had undergone recent liver transplantation (perhaps due to HCC or cirrhosis). However, as the majority of these samples (all except type 1, type 3 and most of type 2b isolates which were obtained from Norweigan patients with chronic

hepatitis C) were obtained from blood donors for which a donor health assessment was unavailable, the likelihood of these individuals being immunocompromised is low. In addition, there was no difference in the frequency of unreactive samples between those originating from blood donors and those from hepatitis patients. An absence of NS4 antibody might also be observed if the individual had a specific inability to make an antibody response towards this epitope, or had only recently been infected and the sample taken prior to seroconversion.

In general, the level of antibody reactivity towards antigens in the 1-6 serotyping assay was lower than that in the 1-3 serotyping assay, and so a 1/20 dilution was used for the 1-6 assay as opposed to 1/40 serum dilution used in the 1-3 assay. The lower level of antibody reactivity to peptides in the 1-6 assay may be due to cross reactivity between competing peptides corresponding to types 1 and 4-6 (Table 3.2.1). This cross reactivity would mean that only antibody specific for region 2 peptides would be able to bind to the solid phase, and so reduce the signal.

The specificity of the 1-6 assay was high, with 97.3% of samples being serotyped in agreement with the genotype identified by RFLP. Although the assay was 100% accurate for the detection of types 1a and 2a, for other types discrepant samples included the detection of type 6 antibody in a patient infected with type 1b, type 1 antibody in a sample genotyped as 2b, type 2 antibody in sera from patients infected with either types 3 or 4 and finally a sample from an individual infected with type 5 which was serotyped as type 1. Discrepant results may be

explained by the existence of unusual variants of HCV in which amino acid variation within regions coding for the peptides used in the assay could lead to cross reactivity with antigen towards heterologous genotypes, or that the consensus sequence used to design peptides for each genotype may not be fully representative of all isolates. This may be the case with the type 6 peptides, which were based on the analysis of only two sequences. Another possible explanation is that the assay has detected circulating antibody induced by a previous infection with a different genotype. This is especially likely in patients with a history of multiple exposure such as haemophiliacs. Finally, discrepant results could arise if an individual were infected with a recombinant virus which contained sequences in the 5' NCR and NS4 specific to different genotypes. However, there is no strong evidence for recombination of HCV. A more detailed study of discrepant results is described in chapter 6.

A high frequency of type 4 samples (14.5%) contained non-type-specific antibodies (9/62 - table 3.2.4) suggesting that cross reactivity with other peptides in competing solutions prevents binding to peptides on the solid phase. Type 4 is one of the most heterogenous genotypes of HCV with numerous subtypes described (a-h) (Simmonds, 1995; Bukh, 1995; Fretz *et al.,* 1995). The type 4 specific peptides used in the assay represent only subtype 4a, and it is quite likely that samples giving non-specific reactivity may be from individuals infected with other subtypes of type 4 with divergent NS4 sequences. Similarly, the difference in the specificity of the ELISA between types 1a and 1b may represent a higher variation

in antigenic regions for 1b than observed in the sequences analysed. The sensitivity and specificity of the assay for genotypes 5 and 6 were both relatively low, but only a few samples were available for serotyping analysis.

3.3 APPLICATION OF THE 1-6 SEROTYPING ELISA.

3.3.1 INTRODUCTION.

After confirmation that the 1-6 serotyping assay was able to successfully distinguish between type-specific antibody produced towards infections with all six HCV genotypes, an investigation was carried out into the performance of the assay when applied to specific populations.

The application of the serotyping assay towards different populations was also carried out to investigate further the epidemiology of HCV, and the genotype distribution in geographical areas for which little or no information had previously been acquired.

3.3.2 SAMPLES.

Samples were obtained from Pakistan, Egypt, Taiwan and Norway.

Pakistan.

A total of 87 samples from patients with chronic hepatitis C in Karachi, Pakistan were collected (Dr. Hasnain Ali-Shah, Department of Medicine, The Aga Khan University, Pakistan) and screened with the Murex anti-HCV enzyme immunoassay VK47/48 for the presence of HCV antibody, and by PCR for the detection of virus RNA. Samples producing negative results with both methods were excluded from the study, leaving a total of 62 sera for further analysis by RFLP of the 5' NCR and serotyping.

Egypt.

52 samples were obtained from patients with chronic hepatitis C in Cairo (Professor A. El-Zayadi, Cairo Liver Centre, Giza, Egypt) and analysed by PCR, RFLP and serotyping using the 1-6 ELISA.

Taiwan.

A set of 49 HCV positive blood donor samples from Taiwan were obtained from a larger study performed by Dr. F. Davidson. Samples had been previously analysed by PCR and the HCV genotype determined by RFLP in those which were positive.

Norway.

A total of 118 samples from Norwegian patients with chronic HCV were collected (Dr. Helge Bell, Aker University Hospital, Oslo), and analysed by PCR and RFLP to determine the genotype of HCV causing infection.

3.3.3 RESULTS.

Pakistan

Virus RNA was amplified in 48/62 HCV antibody positive samples, and RFLP analysis of the 5' NCR revealed genotype 1 in 3 samples (6.25%), type 2 in 1 sample (2.1%), type 3 in 43 samples (89.6%) and type 5 in 1 sample (2.1%).

When all 62 samples were analysed with the 1-6 serotyping assay (Table 3.3a), both the sensitivity and specificity of the ELISA towards type 3 was low.

Type-specific antibody was detected in only 23/43 (52.3%) type 3 samples, while two samples that were type 3 by RFLP were serotyped as type 2 or type 4.

Type specific antibody was detected in 6/14 PCR negative samples, 5 of which were serotyped as type 3. Sequence analysis in the 5' NCR of two samples identified by RFLP as type 3 (one of each producing NTS and NR results on the ELISA) revealed that these variants were more specifically type 3b. RFLP analysis of these samples was carried out by Miss. J. Ellender.

Egypt.

From a total of 51 samples, 35 were PCR positive and could be genotyped by RFLP. Apart from one type 1 sample, all were type 4 (34/35; 97.1%). Type-specific antibody was detected in 37 sera (Table 3.3b), and was in each case concordant with the genotype identified by RFLP. Of 34 samples from individuals infected with type 4, type-specific antibody was detected in 27 (sensitivity 79.4%) with the remaining 7 samples untypeable despite showing reactivity towards NS4 peptides in the unblocked control well of the assay (NTS). Similarly, of the 16 samples which were PCR negative, 8 were serotype 4, 1 was serotype 1 and 3 samples showed non-type-specific reactivity towards NS4 peptides although 4 were unreactive.

Taiwan.

Genotyping of 49 samples from Taiwan by RFLP prior to this study (Davidson 1995) had revealed subtypes 1a (n=2), 1b (n=27), 2a (n=14), 2b (n=4) with 2 samples negative by PCR. The sensitivity of the serotyping assay in this

Table 3.3a DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV PATIENT SAMPLES FROM PAKISTAN USING THE 1-6 SEROTYPING ELISA.

Genotype (RFLP)	Serotype 1	2	3	4	5	6	NTS*	NR†	Total	Sensitivity	Concordance
1	2	-	1	-	-	-	-	-	3	100%	66.7%
2	-	1	-	-	-	-	-	-	1	100%	100%
3	-	1	21	1	-	-	11	9	43	52.3%	91.3%
4	-	-	-	-	-	-	-	-	0	-	-
5	-	-	-	-	1	-	-	-	1	100%	100%
6	-	-	-	-	-	-	-	-	0	-	-
PCR negative	1	-	5	-	-	-	1	7	14	42.9	n/a‡
Total	3	2	27	1	1	0	12	16	62	54.8	89.3¶

* Non-type-specific reactivity (appendix 1.0)
† Non reactive
‡ Not applicable as these samples could not be genotyped
¶ Calculated from the results of samples infected with types 1-6 only

Table 3.3b DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV PATIENT SAMPLES FROM EGYPT USING THE 1-6 SEROTYPING ELISA.

Genotype	Serotype										
(RFLP)	1	2	3	4	5	6	NTS*	NR†	Total	Sensitivity	Concordance
1	1	-	-	-	-	-	-	-	1	100%	100%
2	-	-	-	-	-	-	-	-	0	-	-
3	-	-	-	-	-	-	-	-	0	-	-
4	-	-	-	27	-	-	7	-	34	79.4%	100%
5	-	-	-	-	-	-	-	-	0	-	-
6	-	-	-	-	-	-	-	-	0	-	-
PCR negative	1	-	-	8	-	-	3	4	16	56.3%	n/a‡
Total	2	-	-	35	-	-	10	4	51	72.5%	100%¶

* Non-type-specific reactivity (appendix 1.0)
† Non reactive
‡ Not applicable as these samples could not be genotyped
¶ Calculated from the results from samples infected with types 1-6

study ranged from 77.8% (type 1b) and 78.6% (type 2a) to 100% for types 1a and 2b (Table 3.3c). The only discrepant result was a genotype 2 sample which was serotyped as type 1. Both PCR negative samples were identified as type 1 by serotyping.

Norway.

The genotype distribution in 108 Norwegian samples HCV positive by PCR was 24.1% type 1a, 10.2% 1b, 13.0% 2b, 50.9% 3a and 0.9% type 4. Ten sera were PCR negative.

The overall sensitivity of the serotyping assay was 93.5% (101/108 samples), with the detection of type-specific antibody corresponding to the genotype identified by RFLP in all but 2 samples (Table 3.3d). All but one of the samples which were negative could be serotyped, and consisted of three serotype 1, one serotype 2 and five serotype 3.

3.3.4 DISCUSSION.

In this study, the 1-6 serotyping ELISA was applied to samples obtained from various different populations to investigate the distribution of HCV variants causing infection in these areas, and to estimate the ability of the assay in the detection of type-specific antibody generated towards them.

Prior to this study, the HCV genotype distribution in Pakistan had not been investigated. Infection with type 3 was identified by RFLP in the vast majority of samples (89.6%) with the remainder type 1, 2 or 5. The predominance of type 3

Table 3.3c DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV POSITIVE BLOOD DONOR SAMPLES FROM TAIWAN USING THE 1-6 SEROTYPING ELISA.

Genotype	Serotype										
(RFLP)	1	2	3	4	5	6	NTS*	NR†	Total	Sensitivity	Concordance
1a	2	-	-	-	-	-	-	-	2	100%	100%
1b	21	-	-	-	-	-	4	2	27	77.8%	100%
2a	-	11	-	-	-	-	1	2	14	78.6%	100%
2b	1	3	-	-	-	-	-	-	4	100%	75.0%
PCR negative	2	-	-	-	-	-	-	-	2	100%	n/a‡
Total	26	14	0	0	0	0	5	4	49	81.6%	97.4%¶

* Non-type-specific reactivity (appendix 1.0)
† Non reactive
‡ Not applicable as these samples could not be genotyped
¶ Calculated from the results from samples which could be genotyped

Table 3.3d DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV PATIENT SAMPLES FROM NORWAY USING THE 1-6 SEROTYPING ELISA.

Genotype	Serotype										
(RFLP)	1	2	3	4	5	6	NTS*	NR†	Total	Sensitivity	Concordance
1a	25	-	-	-	-	-	-	1	26	96.2%	100%
1b	9	-	-	-	-	1	-	1	11	90.9%	90.0%
2b	-	14	-	-	-	-	-	-	14	100%	100%
3a	-	1	50	-	-	-	2	2	55	92.7%	98.0%
4	-	-	-	1	-	-	-	1	2	50.0%	100%
PCR negative	3	1	5	-	-	-	1	-	10	90.0%	n/a‡
Total	37	16	55	1	0	0	3	5	118	93.2%	98.0%¶

* Non-type-specific reactivity (appendix 1.0)
† Non reactive
‡ Not applicable as these samples could not be genotyped
¶ Calculated using the results from samples which could be genotyped

in this area has since been confirmed in a study by Tong *et al* in which 15 HCV positive samples were type 3 by RFLP (Tong *et al.,* 1996). The sensitivity of the serotyping assay for type 3 was much lower for isolates originating from Pakistan (sensitivity 52.3%) than for Norway (sensitivity 92.7%) (Table 3.3.4). The type 3 peptides used in the assay are derived from subtype 3a sequences, and the Norwegian patients are likely to be infected with this subtype, as sequence analysis of type 3 isolates in Europe has revealed the majority to be subtype 3a (McOmish *et al.,* 1994; Bukh *et al.,* 1993). In contrast, the existence of novel subtypes of type 3 in Pakistan, India, Bangladesh and Nepal has been established by sequence analysis in other studies (Valliammai *et al.,* 1995; Tokita *et al.,* 1994; Mellor *et al.,* 1995). The relatively low sensitivity of the serotyping assay in Pakistan suggests that the level of amino acid variation between type 3 subtypes may be sufficient to prevent the recognition of type-specific antibody by type 3a peptides alone. Indeed, two of the samples which were untypeable by serotyping were type 3b by sequence analysis. The sensitivity of the serotyping assay to type 3 variants could theoretically be increased by the addition of peptides specific to further subtypes. However, at least seven and possibly as many as seventeen subtypes of type 3 have been described, and so this may not be practical.

The high prevalence of type 4 in Egypt described previously (McOmish *et al.,* 1994; Simmonds *et al.,* 1993) was confirmed in this study. Although nine subtypes of type 4 were identified among a relatively small number of HCV infected individuals in regions of Central Africa (Stuyver *et al.,* 1994; Bukh *et al.,*

Table 3.3.4 A COMPARISON OF THE ABILITY OF THE 1-6 SEROTYPING ASSAY TO DETECT TYPE-SPECIFIC ANTIBODY IN HCV INFECTED INDIVIDUALS FROM DIFFERENT POPULATIONS

		% of samples with reactivity		Performance of Assay	
Population	No. samples	NTS*	NR†	Sensitivity	Concordance‡
Various	210	7.1	5.7	87.1%	97.3%
Pakistan	62	19.4	25.8	54.8%	89.3%
Egypt	51	19.6	7.8	72.5%	100%
Taiwan	49	10.2	8.2	81.6%	97.4%
Norway	118	2.5	4.2	93.2%	98.0%

* Non-type-specific reactivity (appendix 1.0)
† Non reactive
‡ Concordance (appendix 1.0) of serotyping results with genotyping by RFLP of the 5' NCR. Calculations represent results from PCR positive sera only.

1994; Bukh *et al.,* 1993), the majority of type 4 infections in Egypt are thought to be caused by type 4a. The serotyping of 79.4% type 4 Egyptian samples using type 4a peptides is consistent with this although the inability to serotype seven genotype 4 samples suggests the presence of additional subtypes.

The serotyping ELISA was able to detect extra genotypes not previously observed in some of the study groups, for example, type 4 in Norway and type 5 in Pakistan. Risk factors were not available for these individuals, but it is likely that these infections have been acquired elsewhere, perhaps if the individual was an immigrant. Although the different HCV genotypes display specific geographical distributions, the recent increase in world travel and immigration has tended to break down these differences. For example, type 4 infections have been described in the U.S.A. (Lau *et al.,* 1996), types 4, 5 and 6 in France (Stuyver *et al.,* 1996a; Pawlotsky *et al.,* 1997a) and type 5 in Ireland (Chamberlain *et al.,* 1997), so the assay should be efficient not only in the identification of genotypes commonly found in a specific region but also in the isolation of unusual variants within a larger population. Certain genotyping methods, for example the use of type-specific primers for the amplification of virus DNA by PCR (Okamoto *et al.,* 1990; Okamoto *et al.,* 1992), are designed to be able to identify genotypes present in a specific area, and will therefore be unable to recognise unusual variants, or will identify them incorrectly.

Although the addition of peptides specific for certain HCV subtypes (1a, 1b, 2a, 2b, 2c) to the assay has increased the sensitivity of the ELISA towards these

isolates, the level of antigenic variation between these subtypes is too low for the assay to be able to distinguish between them. Although certain genotyping methods are able to distinguish between some HCV subtypes, for example types 1a/b, 2a/b and 3a/b by RFLP of the 5' NCR (Davidson *et al.,* 1995) and 1a/b, 2a/b by the line probe assay (Stuyver *et al.,* 1996b), problems may still be encountered. The nucleotide sequences for types 2a/2c in the 5' NCR are too similar to allow these subtypes to be distinguished by either method, and similarly, the identity between novel subtypes of type 6 and type 1 in this region has prevented the identification of these isolates by such assays (discussed in chapter 5).

CHAPTER 4

4 THE ROLE OF NS4 ANTIGENIC REGION 3 IN HCV SEROTYPING.

4.1 GENERAL INTRODUCTION.

The previous chapter has described how the prototype serotyping ELISA for HCV genotypes 1-3 using synthetic peptides corresponding to two antigenic regions in NS4, was successfully extended to identify antibody to all six genotypes. Although the sensitivity and specificity of the 1-6 serotyping assay was high, differences in reactivity were observed for individual genotypes. Type-specific antibody was detected in 80.6%, 68.8% and 66.6% samples infected with types 4, 5 and 6 respectively compared with 92.3%, 96.9% and 92.7% for samples infected with types 1, 2 and 3 (chapter 3; Table 3.2.4.). A comparison of NS4 sequences for all six genotypes (chapter 3; Figure 3.2.1) revealed similarities between region 1 peptides of types 1, 4, 5 and 6, suggesting that cross-reactivity in region 1 may account for the reduced sensitivity of the assay for types 4-6 since successful serotyping would therefore rely on antibody produced towards antigenic region 2.

In order to overcome this problem, the possibility of including a third antigenic region was investigated.

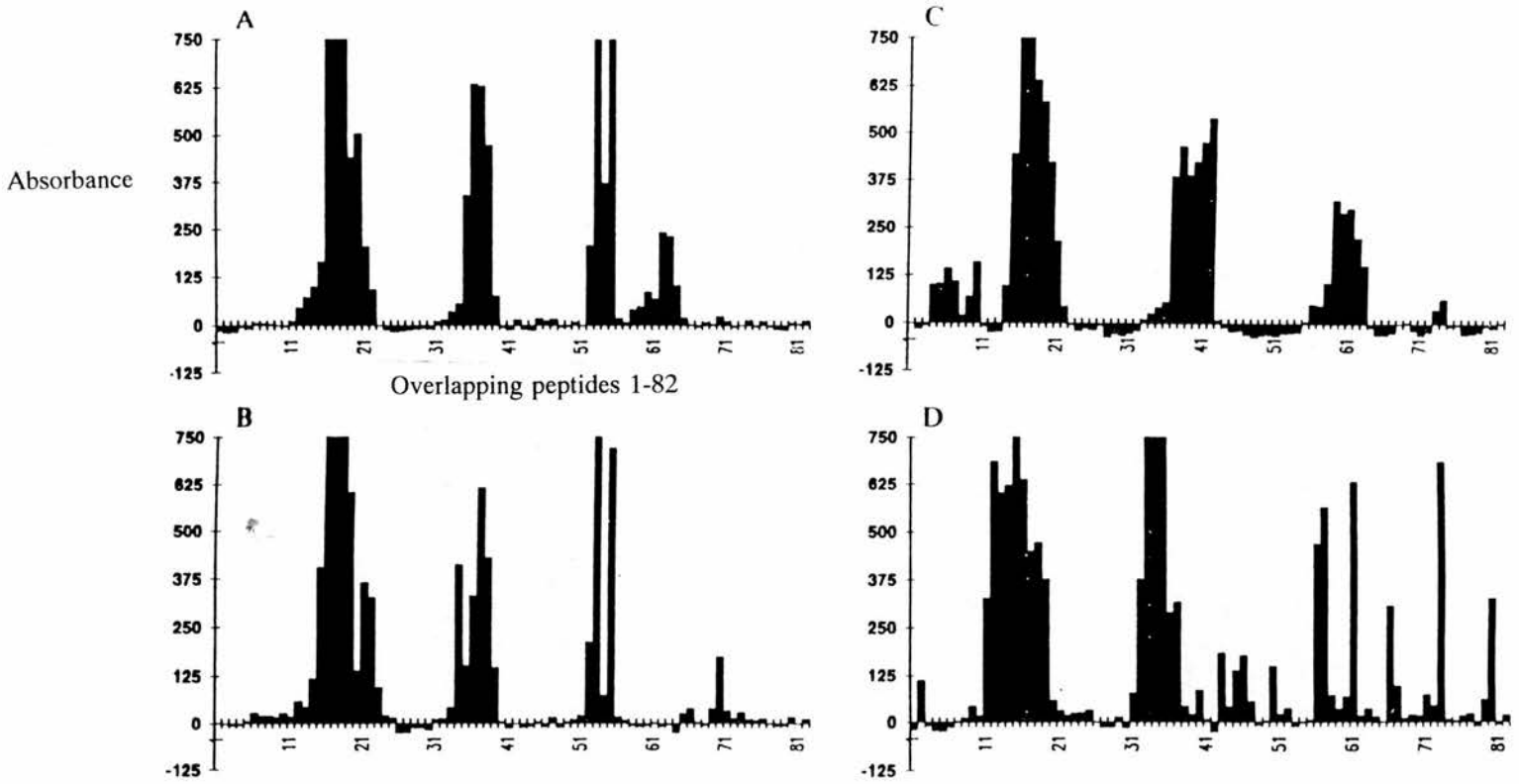
4.2 SYNTHETIC PEPTIDES FOR ANTIGENIC REGION 3.

Epitope mapping of the NS4 region (Simmonds *et al.,* 1993d) was previously carried out using a set of 82 overlapping nonapeptides from amino acid

positions 1679-1768, each overlapping the preceding peptide by eight residues. Antibody reactivity to individual peptides revealed two major antigenic regions, spanning amino acid positions 1691-1708 (region 1) and 1720-1738 (region 2). Reactivity towards a third region was also detected (Figure 4.2) which covered a less defined area, ranging from amino acid positions 1743-1755 in type 1b while extending to position 1763 in types 1a and 2b, but with only intermittent peaks of reactivity towards peptides corresponding to type 3 sequences. A comparison of NS4 sequences (chapter 3; Figure 3.2.1) revealed significant levels of amino acid variation between the six major genotypes, and consequently peptides spanning region 3 were synthesised (corresponding to amino acids 1738-1757) for genotypes 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 4, 5 and 6 (Table 4.2).

Region 3 peptides were incorporated into the serotyping ELISA in three ways; (i) regions 1 and 3, (ii) regions 2 and 3 and (iii) regions 1, 2 and 3, and the antibody reactivity to each compared with that observed with the standard assay using regions 1 and 2. Concentrations of peptides used both for coating microtitre plates and in competing peptide solutions were identical for each version of the assay, as was the concentration of serum used for each test. All proceedures and conditions for each assay, such as incubation times and temperatures, were also kept constant.

Figure 4.2 ANTIGENIC REGIONS IN NS4 REVEALED BY EPITOPE MAPPING



Reactivity of anti-HCV positive samples with overlapping peptides numbered 1-82. Reactivity of samples from an HCV-type 1 infected individual (A and B) with peptides of subtype 1a (A) or 1b (B); reactivity of a type 2 infected donor (C) with type 2b peptides; reactivity of a type 3 infected donor (D) with type 3 peptides. O.D.s are expressed in milliunits in the range of 125-750mU.
Antigenic region 1 = peptides 13-22 (amino acids 1691-1708)
Antigenic region 2 = peptides 32-42 (amino acids 1720-1738) **Figure reproduced from Simmonds *et al.*, 1993.**

Table 4.2 AMINO ACID SEQUENCES FOR NS4 PEPTIDES IN ANTIGENIC REGION 3

HCV Genotype	Peptide Sequence (amino acids 1738-1757)
1a	TASRQAEVIAPAVQTNWQKL
1b	..TK....A..VIESK.RA.
1c	...K...AAV.V..S....V
2a	Q..K..QD.Q....AS.P.V
2b	Q.T...QD.Q..I.SS.P..
2c	Q..K..QD.Q.V..GT.P..
3a	R.TQ.QA..E.I.A.....
3b	R..H.EAD.E.I.QTN...V
4	L.GKAEQEAT.VI.S.FA..
5	..GQK..TLK..ATSM.NRA
6	ASAQ...ELK...HSA.P.M

"." Sequence identity to type 1a sequence

4.3 SAMPLES.

Samples were as described for chapter 3, section 3.3.2 and were from Norway (type 1a, n=10; type 1b, n=10; type 2b, n=10; type 3, n=20), Taiwan (type 2a, n=9) or Egypt (type 4, n=20). Samples from individuals infected with types 5 (n=5) or 6 (n=2) had also been used previously in the evaluation of the serotyping ELISA (section 3.2.3).

Where sufficient volumes of serum were available, each sample was tested in triplicate using all four versions of the assay. The serotype of antibody within each sample was inferred from the mean of the absorbance readings from all three results.

4.4 RESULTS

Significant differences were observed between the overall sensitivity of each assay for all virus genotypes and also in the serological reactivity induced by individual virus genotypes to different combinations of NS4 peptides. The serotyping ELISA that used antigenic regions 1 and 3 was by far the least efficient of the four assays, with type-specific antibody being detected in only 42.9% samples tested (Table 4.4a). Of 18 type 4 samples, the only three sera that were typeable using peptides designed towards regions 1 or 3, were mistyped, and the majority of type 4 samples (14/18) displayed non-type-specific reactivity. The sensitivity of the 1+3 assay for other genotypes was also low, ranging from 0% (type 6) to 65% (type 3). However, the type-specific antibody detected in samples

from individuals infected with types 1a, 1b, 2a, 2b and 5 were all concordant with the genotype identified by RFLP analysis.

In contrast, the other three versions of the ELISA (regions 2 and 3, regions 1, 2 and 3 and the standard assay using regions 1 and 2) were all more sensitive. The overall sensitivity of the assay based on regions 2 and 3 (Table 4.4b) was 75.6%, although this assay was only able to detect type-specific antibody in 4/9 (44.4%) samples from individuals infected with type 2a in comparison with 5/9 samples for the 1+3 assay. On the other hand, the 2+3 assay detected type-specific antibody in 100% of samples from individuals infected with type 1a (regions 1 and 3; 40%) and 85% of samples from individuals infected with type 4 (region 1 and 3; 16.7%). Serotyping using regions 2 and 3 was accurate in the majority of cases except for type 3 samples, where two discrepant results were observed with reactivity towards type 1 and type 5 peptides, while one sample from an individual infected with type 1b was identified as type 6.

The overall performance of assays using antigens derived from regions 1 and 2 (Table 4.4d), or from all three regions (Table 4.4c) was identical, although there were slight differences in the sensitivity and specificity towards individual genotypes. The presence of region 3 peptides seemed to enhance type-specific reactivity for one sample infected with type 2a, but reduced reactivity for single samples from individuals infected with type 2b or type 3. The significance of region 3 peptides for the detection of type 6-specific antibody was difficult to determine, as only two samples with sufficient volumes were available. However,

Table 4.4a DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV POSITIVE SAMPLES USING A SEROTYPING ELISA BASED ON NS4 PEPTIDES FROM ANTIGENIC REGIONS 1 AND 3

Genotype (RFLP)	Serotype 1	2	3	4	5	6	NTS*	NR†	Total	Sensitivity	Concordance
1a	4	-	-	-	-	-	5	1	10	40%	100%
1b	3	-	-	-	-	-	4	3	10	30%	100%
2a	-	5	-	-	-	-	2	2	9	55.6%	100%
b	-	6	-	-	-	-	2	2	10	60%	100%
3	-	-	12	-	-	1	4	3	20	65%	92.3%
4	1	2	-	-	-	-	14	1	18	16.7%	0%
5	-	-	-	-	1	-	5	-	5	20%	100%
6	-	-	-	-	-	-	2	-	2	0%	n/a
Total							38	12	84	42.9%	88.9%

Table 4.4b DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV POSITIVE SAMPLES USING A SEROTYPING ELISA BASED ON NS4 PEPTIDES FROM ANTIGENIC REGIONS 2 AND 3

Genotype (RFLP)	Serotype 1	2	3	4	5	6	NTS	NR	Total	Sensitivity	Concordance
1a	10	-	-	-	-	-	-	-	10	100%	100%
1b	7	-	-	-	-	1	-	2	10	80%	87.5%
2a	-	4	-	-	-	-	3	2	9	44.4%	100%
2b	-	9	-	-	-	-	1	-	10	90%	100%
3	1	-	12	-	1	-	5	1	20	70%	85.7%
4	-	-	-	17	-	-	-	3	20	85%	100%
5	-	-	-	-	2	-	-	3	5	40%	100%
6	-	-	-	-	-	1	-	1	2	50%	100%
Total							9	12	86	75.6%	95.4%

* Non-type-specific reactivity (appendix 1.0)

† Non reactive

Table 4.4c DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV POSITIVE SAMPLES USING A SEROTYPING ELISA BASED ON NS4 PEPTIDES FROM ANTIGENIC REGIONS 1, 2 AND 3

Genotype (RFLP)	Serotype 1	2	3	4	5	6	NTS*	NR†	Total	Sensitivity	Concordance
1a	10	-	-	-	-	-	-	-	10	100%	100%
1b	7	-	-	-	-	1	1	1	10	80%	87.5%
2a	-	9	-	-	-	-	-	-	9	100%	100%
2b	-	9	-	-	-	-	1	-	10	90%	100%
3	-	-	14	-	-	1	3	2	20	75%	93.3%
4	-	-	-	16	-	-	4	-	20	80%	100%
5	-	-	-	-	2	-	2	1	5	40%	100%
6	-	-	-	-	-	1	1	-	2	50%	100%
Total							12	4	86	81.4%	97.1%

Table 4.4d DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV POSITIVE SAMPLES USING A SEROTYPING ELISA BASED ON NS4 PEPTIDES FROM ANTIGENIC REGIONS 1 AND 2

Genotype (RFLP)	Serotype 1	2	3	4	5	6	NTS	NR	Total	Sensitivity	Concordance
1a	10	-	-	-	-	-	-	-	10	100%	100%
1b	7	-	-	-	-	1	1	1	10	80%	87.5%
2a	-	8	-	-	-	-	1	-	9	88.9%	100%
2b	-	10	-	-	-	-	-	-	10	100%	100%
3	-	-	15	-	-	1	1	3	20	80%	93.8%
4	-	-	-	16	-	-	4	-	20	80%	100%
5	-	-	-	-	2	-	2	1	5	40%	100%
6	-	-	-	-	-	-	-	2	2	0%	n/a
Total							9	7	86	81.4%	97.1%

* Non-type-specific antibody (appendix 1.0)
† Non reactive

Table 4.5 A COMPARISON OF ABSORBANCE VALUES FROM INDIVIDUAL SAMPLES PRODUCING DIFFERENT RESULTS WITH FOUR VERSIONS OF THE SEROTYPING ASSAY

Assay	UB*	FB†	Well on Microtitre plate 1	2	3	4	5	6	Result
Type 1a - Sample N4									
1+2	1.696	0.057	1.800	0.061	0.068	0.082	0.080	0.094	1
1+3	1.202	0.018	0.034	0.030	0.034	0.014	0.012	0.023	NTS‡
2+3	0.739	0.062	1.786	0.103	0.100	0.074	0.138	0.093	1
1+2+3	1.738	0.027	1.659	0.063	0.049	0.063	0.047	0.095	1
Type 1b - Sample N17									
1+2	1.158	0.032	1.302	0.028	0.024	0.031	0.050	0.051	1
1+3	0.044	0.006	0.007	0.029	0.003	0.007	0.000	0.016	NR¶
2+3	1.464	0.050	1.379	0.029	0.041	0.118	0.076	0.216	1
1+2+3	1.031	0.005	1.304	0.024	0.004	0.016	0.009	0.015	1
Type 2a - Sample T3									
1+2	1.771	0.028	0.049	1.741	0.078	0.087	0.050	0.038	2
1+3	1.853	0.025	0.044	1.782	0.037	0.052	0.046	0.025	2
2+3	0.014	0.013	0.011	0.018	0.002	0.004	0.016	0.020	NR
1+2+3	1.811	0.046	0.095	1.728	0.118	0.175	0.132	0.113	2
Type 2b - Sample N2									
1+2	0.508	0.017	0.022	0.538	0.023	0.026	0.015	0.015	2
1+3	0.012	0.012	0.036	0.037	0.036	0.033	0.027	0.033	NR
2+3	0.775	0.039	0.050	0.700	0.026	0.021	0.045	0.072	2
1+2+3	1.059	0.016	0.003	0.963	0.017	0.017	0.011	0.005	2
Type 3a - Sample N8									
1+2	1.812	0.170	0.210	0.147	1.801	0.055	0.035	0.044	3
1+3	1.760	0.019	0.046	0.020	1.733	0.042	0.053	0.025	3
2+3	1.057	0.060	0.140	0.081	0.932	0.061	0.041	0.057	3
1+2+3	1.809	0.146	0.165	0.169	1.799	0.201	0.111	0.083	3
Type 4 - Sample EG3									
1+2	1.932	0.294	0.347	0.446	0.228	1.789	0.222	0.277	4
1+3	1.757	0.046	0.169	0.067	0.087	0.100	0.150	0.139	NTS
2+3	1.864	0.438	0.062	0.067	0.218	1.830	0.182	0.334	4
1+2+3	1.803	0.232	0.307	0.227	0.262	1.780	0.309	0.259	4
Type 5 - Sample C1016116									
1+2	1.529	0.370	0.444	0.383	0.318	0.416	1.837	0.362	5
1+3	1.878	0.127	0.117	0.164	0.197	0.094	0.175	0.112	NTS
2+3	1.611	0.228	0.404	0.449	0.293	0.330	1.563	0.269	5
1+2+3	1.779	0.266	0.441	0.383	0.332	0.303	1.730	0.368	5
Type 6 - Sample T3950									
1+2	0.186	0.039	0.029	0.058	0.032	0.030	0.040	0.128	6
1+3	0.757	0.042	0.072	0.051	0.124	0.046	0.035	0.041	NTS
2+3	0.132	0.024	0.030	0.042	0.029	0.021	0.022	0.116	6
1+2+3	0.215	0.028	0.030	0.038	0.020	0.038	0.037	0.113	6

* Unblocked positive control well
† Fully blocked negative control well
‡ Non-type specific reactivity
¶ Non reactive

one of these samples was typeable by the assays which included regions 2 and 3 together.

4.5 DISCUSSION.

Despite the ability of the 1-6 serotyping ELISA to detect type-specific antibody in samples from HCV infected patients from various geographical locations, the extensive heterogeneity observed between different isolates creates a constant challenge for the improvement of assay sensitivity and specificity. One reason for the lower sensitivity of the 1-6 assay observed for types 4-6 in some studies (Bhattacherjee *et al.*, 1995) could be amino acid sequence similarity between these genotypes and type 1 in NS4 antigenic region 1.

A panel of samples was analysed using four versions of the assay, consisting of each possible combination of peptides for three antigenic regions of NS4. The results indicate that the presence of peptides corresponding to antigenic region 2 of NS4 is essential for the discrimination between type-specific antibody induced by different HCV genotypes. The sensitivity of the assay based on regions 1 and 3 alone was only 42.9%, compared with 75.6%-81.4% observed using other combinations of antigenic regions but including region 2. Type 6-specific antibody was only detected in the presence of both antigenic regions 2 and 3, although only two samples were available and further studies using additional samples would be necessary to confirm this.

A detailed comparison of absorbance values in individual typing wells for the same sample in different versions of the assay revealed that type-specific reactivity was often dependent on only one antigenic region (Table 4.5). For example samples N4, N17, N2, EG3, C1016116 and T3950 were only typeable using versions of the assay which included antigenic region 2. Similarly, antibody specific to type 2a was dependent upon the presence of antigenic region 1 peptides in sample T3, as the sensitivity of the assay based on regions 2 and 3 was only 44.4%. In samples where serotyping was dependent on region 2 alone, antibody generated towards antigenic region 1 or 3 might be present in the serum but remain undetected because of to cross reactivity with heterologous competing peptides. Such cross reactivity may occur between type 1 peptides for region 1 and those for types 4-6 because of their relatively high level of amino acid sequence identity, but cross reactivity seems unlikely for antigenic region 3 where the level of identity is lower.

The specificity of all versions of the serotyping assays was relatively high, with the lowest value observed for the regions 1+3 assay (88.9%). In the absence of region 2 the majority of samples infected with type 4 (83.3%) were untypeable, and the three samples in which type-specific antibody could be detected were identified as either type 1 or type 2. The amino acid sequence "QPAV" is present both at the beginning of the genotype 4 region 1 peptide, and within the type 2a region 3 peptide and cross-reactivity between these peptides may contribute towards the misidentification of type 4 samples. The large number of type 4 samples with

"NTS" reactivity with the 1+3 assay confirms the suspicion that region 1 peptides for type 4 cross react with those for type 1, and that an antibody response to region 3 is either rare, or also lacking in specificity. Similarly, the difference in the sensitivity of the 1+3 assay towards different HCV genotypes may reflect the amount of sequence similarity between type 1 and type 4-6 peptides for antigenic region 1, as type-specific antibody could be detected in 55-65% samples infected with types 2a, 2b or 3, compared with 0-40% samples infected with types 1, 4, 5 or 6.

One of the major disadvantages of the 1-6 serotyping assay in comparison with many PCR-based genotyping assays is the inability to distinguish between different subtypes of HCV. A number of groups have suggested that a greater pathogenicity (Hatzakis *et al.,* 1996; Tanaka *et al.,* 1996), and a lower rate of sustained response to interferon therapy (Pawlotsky *et al.,* 1996; Martinot Peignoux *et al.,* 1995; Tsubota *et al.,* 1994) may be associated not simply with infections caused by type 1, but specifically by type 1b. If these associations are confirmed, the importance of subtyping would be greatly increased. The type 1 specific peptides for antigenic region 3 display a number of subtype-specific amino acid motifs (Table 4.2), which could possibly be exploited in an ELISA for subtyping. However, the low frequency of antibody reactivity to region 3 peptides observed in this study suggests that the sensitivity of such an assay would be low.

Since no significant improvement was observed in the sensitivity and specificity of the serotyping ELISA with the addition of peptides for antigenic

region 3, the design of the assay was maintained with peptides for antigenic regions 1 and 2.

CHAPTER 5

5 HCV INFECTION IN HONG KONG; PROPERTIES OF GENOTYPE 6.

5.1 GENERAL INTRODUCTION.

The discovery of unusual HCV variants in samples from Hong Kong, which contained a characteristic 2bp insertion in the 5' NCR at position -143 (Simmonds *et al.,* 1993c; Bukh *et al.,* 1992c) preceded the classification of these variants based on sequence analysis of NS5 as a new major virus genotype (type 6a) (Simmonds *et al.,* 1993b). Geographical surveys of the distribution of HCV genotypes revealed that type 6a infections were common only in South East Asia (Tokita *et al.,* 1994a; Davidson *et al.,* 1995a; McOmish *et al.,* 1994a). The restricted distribution of this genotype has limited the number of samples available for analysis. Although a few sequences have been obtained for E1 (Bukh *et al.,* 1993) and core (Zhang *et al.,* 1995; Bukh *et al.,* 1994), the level of genetic heterogeneity within type 6a, and the existence of additional subtypes has been relatively unexplored.

More recently, several groups have reported the existence of novel genotypes related to type 6a of countries in South East Asia such as Thailand and Vietnam (Tokita *et al.,* 1994a; Mellor *et al.,* 1995a; Apichartpiyakul *et al.,* 1994a). Although these variants are closely related to type 6a, the levels of sequence variability between these viruses is higher than observed between subtypes of other HCV genotypes. For this reason, the classification of these variants as either additional major genotypes 7, 8 and 9 (Tokita *et al.,* 1995; Tokita *et al.,* 1994), or

as diverse subtypes of type 6 (Delamballerie *et al.,* 1997; Simmonds *et al.,* 1996; Mizokami *et al.,* 1996) has been a matter of debate. Although the coexistence of type 6 group variants with type 6a has been confirmed in Vietnam (Tokita *et al.,* 1994a), the presence of these variants in Hong Kong has yet to be investigated.

The availability of a large set of samples from blood donors in Hong Kong allowed an extensive study of both the sequence variation within HCV type 6 and clinical features associated with infection with this genotype.

5.2 IDENTIFICATION OF HCV INFECTION IN BLOOD DONORS FROM HONG KONG.

5.2.1 INTRODUCTION

There are several reports on the prevalence of HCV infection in different groups of individuals from Hong Kong. HCV antibody was detected in 0.5% samples from individuals in the general population (n=910), compared with 70% for a cohort consisting of intravenous drug users, haemophiliacs and patients with chronic NANB hepatitis (Chan *et al.,* 1992). Similarly, the prevalence of anti-HCV among blood donors in Hong Kong was reported as 0.4% (Lin *et al.,* 1992), although this study group was restricted to donors who were negative for hepatitis B surface antigen (HBsAg). A larger study of 29,000 blood donors confirmed the presence of HCV RNA by RT-PCR in 0.13% subjects tested (McOmish *et al.,* 1994). The number of HCV viraemic blood donors in this study was almost double

that among Scottish blood donors (0.07%) but less than that in other countries such as Egypt (8.7%) and Taiwan (1.7%) (McOmish *et al.,* 1994).

5.2.2 SAMPLES

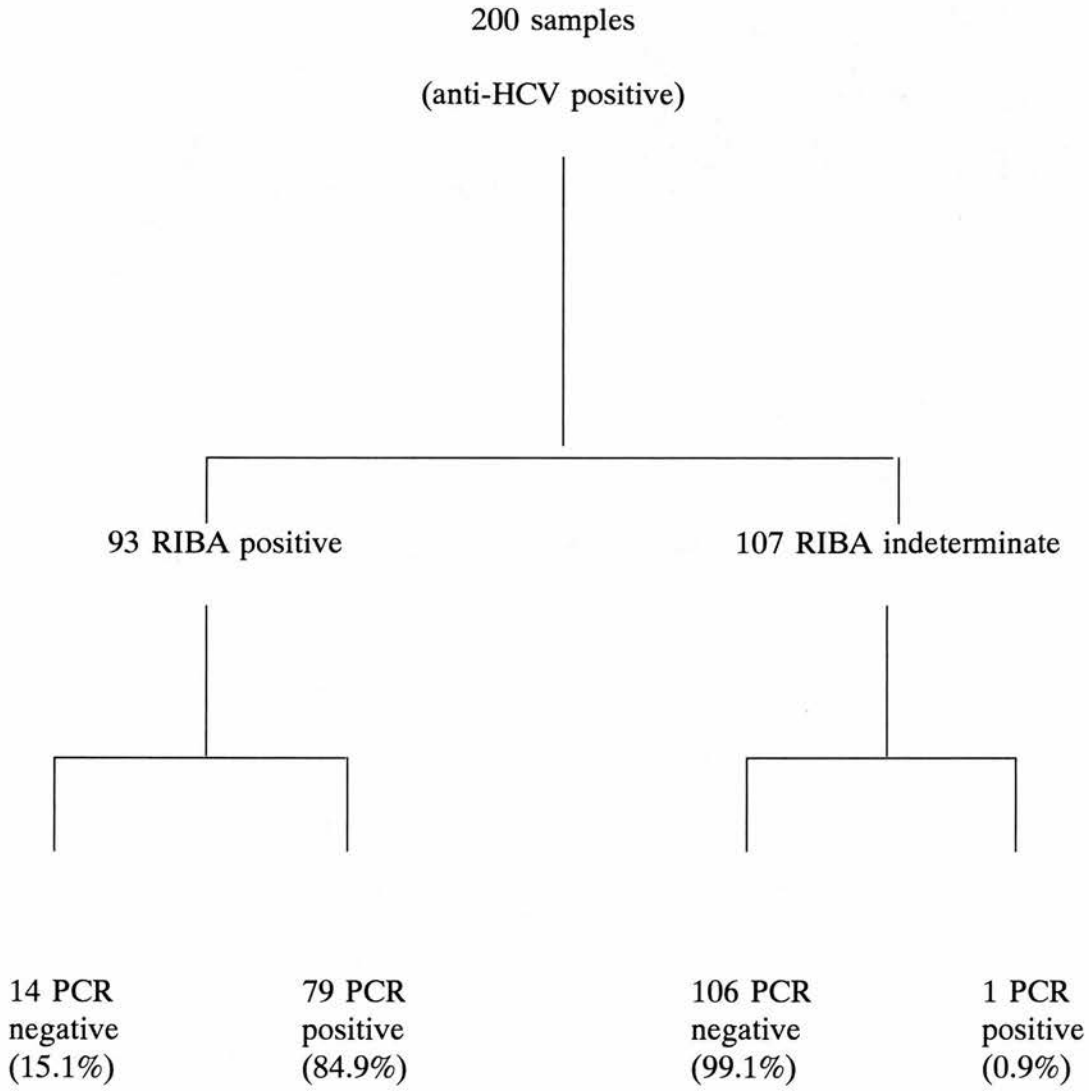
Samples were obtained from voluntary blood donors attending the Hong Kong Red Cross Blood Transfusion Service in 1991-1994. 900 samples that were repeatedly reactive using the Abbott 2nd generation EIA, and were either positive or indeterminate upon supplementary testing with the Abbott Matrix 2nd generation recombinant immunoblot assay (RIBA 2.0) were analysed in this study.

In this study, the detection of HCV RNA in anti-HCV positive samples selected from blood donors in Hong Kong was carried out using the RT-PCR, and the presence of HCV antibody confirmed using a third generation enzyme-immunoassay (EIA) (Murex VK48).

5.2.3 RESULTS

Each sample was re-tested with the Murex anti-HCV screening ELISA producing similar results to those obtained with the Abbott 2nd generation assay used for routine analysis of donations in Hong Kong. Analysis of the first 200 samples revealed that absorbance values for samples with indeterminate RIBA reactivity, were lower than those in samples that were unequivocally positive by RIBA.

Figure 5.2.3 CORRELATION BETWEEN HCV VIRAEMIA AND RIBA REACTIVITY IN BLOOD DONOR SAMPLES FROM HONG KONG



HCV RNA was detected by PCR of the 5'NCR in 80/200 samples. A breakdown of results for the 200 anti-HCV positive samples tested revealed that the majority of viraemic donors (79/80) were also RIBA positive (Figure 5.2.3). HCV RNA was detected in 79/93 (84.9%) RIBA positive samples, but in only 1/107 (0.9%) samples with indeterminate RIBA reactivity. This single PCR positive, RIBA indeterminate sample was also negative with the Murex anti-HCV screening ELISA and was from a donor with a normal ALT level (17.1U/L).

5.2.4 DISCUSSION

Because anti-HCV screening assays often produce false positive results, the capability of supplementary tests such as RIBA for the confirmation of infection with HCV needs to be high. The consistently higher absorbance values on the Murex anti-HCV ELISA with RIBA positive samples was expected, since the antigens used in each assay are similar (Core, NS3 and NS4).

The Abbott Matrix RIBA supplementary test used in this study was able to discriminate between the majority of viraemic blood donors, with 79/80 PCR positive samples confirmed by the assay, and only one PCR positive sample RIBA indeterminate. However, 15.1% of RIBA-confirmed samples were PCR negative, and are likely to represent donors who have cleared virus infection but have circulating HCV antibody. All but one of the samples with indeterminate RIBA status were negative by PCR, and may therefore represent non specific cross-reactivity towards antigens in the assay. Alternatively, these samples may have

come from individuals who cleared HCV infection long ago and in whom the level of circulating antibody has fallen. One PCR positive sample was positive by Abbott anti-HCV ELISA and displayed indeterminate RIBA reactivity but gave a negative result with the Murex anti-HCV assay. This was from a donor with a normal ALT level. This donor may have been infected recently, and undergoing seroconversion at the time when the sample was collected. HCV infected blood taken before seroconversion will not be detected by serological screening assays. Blood taken from a donor in this situation creates a risk that infected blood will be transfused. The only safeguard against HCV transmission from this type of sample is the routine screening of all donations by PCR, which under current technologies is expensive and time consuming. However, since plasma pool screening by PCR in the U.K. and Europe becomes mandatory in April 1998, there is a pressure to develop automated PCR screening methodology that can be used routinely in blood donor centres. Routine donor screening by HCV PCR has already been initiated in Germany and is under development in the U.K.

5.3 IDENTIFICATION OF HCV GENOTYPES IN HONG KONG

5.3.1 INTRODUCTION

The study of the geographical distribution of HCV genotypes has had important implications for many aspects of HCV research and clinical practice, including epidemiological studies (Power *et al.,* 1995), the prediction of disease outcome (Zein *et al.,* 1996) and the efficient detection of the virus in infected individuals (Salmeron *et al.,* 1996). Although a number of different genotyping assays have been described, these are often only able to detect genotypes which are broadly distributed around the world such as types 1, 2 and 3 (Smith *et al.,* 1995a). Such assays would be inappropriate in populations where genotypes 4, 5 and 6 are prevalent. A previous study using RFLP of sequences amplified from the HCV 5' NCR has revealed that type 6a is responsible for nearly one third of HCV infections in Hong Kong blood donors (McOmish *et al.,* 1994). Other reports have described the presence of type 6a in neighbouring countries such as Macau and Vietnam, where it accounts for 22% and 19% of infections respectively (Tokita *et al.,* 1994a; Davidson *et al.,* 1995a; Tokita *et al.,* 1995a).

Recently, variants of type 6 have been identified in South East Asia, that may have 5' NCR sequences identical to those present in types 1a or 1b (Mellor *et al.,* 1995; Tokita *et al.,* 1994), and which may therefore have been typed incorrectly in previous studies which have relied on assays based on the 5' NCR. Two additional RFLP assays have recently been developed, which provide a

method for distinguishing between these type 6 variants and type 1 in both the 5' NCR and core (Mellor *et al.,* 1996). Restriction enzyme digestion of 5' NCR sequences with *Dde* I/*Hpa* II (because of single base polymorphisms present in some variants of the type 6 group), produces distinct patterns with type 1 isolates (pattern A), type 6a (pattern D) and type 6 group variants (patterns B and C). Other variants of the type 6 group which have identical 5' NCR sequences to types 1a or 1b can be identified by RFLP analysis of the core region. Cleavage with the enzymes *Ava* I and *Sma* I in separate reactions results in distinct patterns of fragments for type 1 sequences (type 1a - a1s1 or a2s1; type 1b - a3s1 or a4s5) from those produced by members of the type 6 group (type 6a - a12s5; type 6 group variants - a10s1, a12s5 or a13s5) (Mellor *et al.,* 1996).

The presence of type 6 group variant viruses was investigated in Hong Kong using RFLP analysis based on 5' NCR and core regions, and sequence analysis.

5.3.2 SAMPLES

From 264 RIBA confirmed and 107 RIBA indeterminate samples obtained from blood donors in Hong Kong (section 5.2), a total of 212 that were PCR positive (211/264 RIBA confirmed and 1/107 RIBA indeterminate) were genotyped. All samples were analysed using the three versions of the RFLP assay in the 5' NCR; (1) enzymes *Rsa* I/*Hae* III and *Mva* I/*Hinf* I for the identification of genotypes 1-6 (McOmish *et al.,* 1994; Davidson *et al.,* 1995) (2) identification of

subtypes 1a/1b using *Bst* UI and 2a/2b, 3a/3b using *Scr* FI (Davidson *et al.,* 1995), and (3) differentiation between type 1 sequences and certain type 6 group variants using a combination of the enzymes *Dde* I/*Hpa* II (Mellor *et al.,* 1996). All type 1 or type 6 samples (n=73) were also analysed with a core based RFLP assay for the presence of type 6 group variants.

5.3.3 RESULTS

In order to investigate the presence of type 6 group variants in HCV positive blood donors from Hong Kong, a subset of 73 samples were initially genotyped (and subtyped) by RFLP analysis of the 5' NCR (McOmish *et al.,* 1994; Davidson *et al.,* 1995). Samples provisionally identified as either types 1 or 6 by this method were subjected to two additional enzyme digestion reactions (*Dde* I/*Hpa* II in the 5' NCR; *Ava* I and *Sma* I in core) to enable sequences corresponding to the novel type 6 variants to be distinguished (Mellor *et al.,* 1995).

All samples except two produced recognisable restriction patterns upon digestion with *Rsa* I/*Hae* III and *Mva* I/*Hinf* I, allowing the genotype to be identified (Table 5.3.3a). Two samples produced unusual combinations of restriction patterns (dA and eA). Subtyping using enzyme *Bst* UI (type 1 samples) or *Scr* fI (type 2 or 3 samples) confirmed the presence of types 1a, 1b, 2a, 2b and 6.

All samples with a type 1 RFLP pattern in the 5'NCR were further analysed using *Dde* I/*Hpa* II and consistently produced pattern A, confirming that none of

TABLE 5.3.3a IDENTIFICATION OF GENOTYPES BY RFLP AND SEQUENCING IN THE 5' NCR AND CORE REGIONS

		5' NCR†						CORE†				
Presumed Genotype	No.*	HaeIII/ RsaI	MvaI/ HinfI	BstUI	ScrfI	DdeI/ HpaII	Inferred Genotype	RFLP SmaI	RFLP AvaI	Inferred Genotype	SEQUENCING No.	SEQUENCING Type
Type 1a	3	b	A	A	-	A	1a	s1	a1	1a	-	-
	1	b	A	A	-	A	1a	s5	a1	1a	1	1a
Type 1b	41	b	A	B	-	A	1b	s1	a3	1b	9	1b
	1	d	A	B	-	A	1b	s1	a3	1b	1	1b
	1	e	A	B	-	A	1b	s1	a3	-	-	-
Type 2a	3	c	D	-	D	-	2a	-	-		2	2a
Type 2b	2	d	D	-	F	-	2b	-	-		-	-
Type 6a	21	h	B	-	-	D	6a	s5	a12	6a	20	6a

* Number of samples

† Restriction patterns are classified according to previous analyses, i.e. 5' NCR; Davidson *et al.*, 1995
Core; Mellor *et al.*, 1996

Figure 5.3.3a CLEAVAGE PATTERNS PRODUCED WITH TYPES 1 AND 6 WITH ENZYMES *Dde* I/*Hpa*II.

A	67	27	12	18	127	
B	67	27	12	145		
C	67	27	12	18	25	102
D	67	27	12	18	130	
E	67	27	12	148		
F	67	27	12	18	28	102

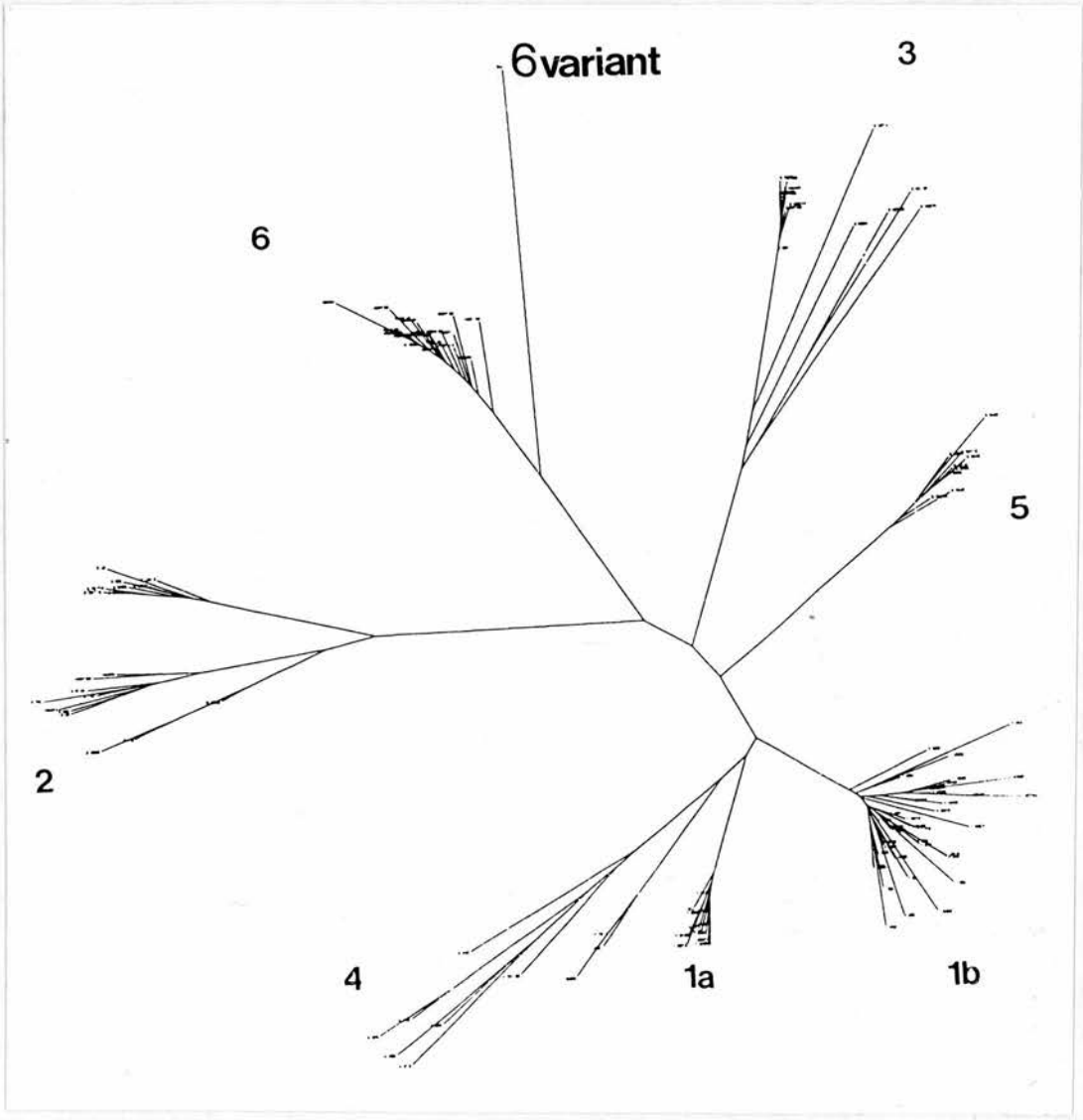
Cleavage sites are indicated by vertical lines.
Numbers represent the sizes of DNA fragments in base pairs.
Patterns A-D are as described previously in Mellor *et al.*, 1996.i.e.A=type 1a, B and C represent type 6 group variants, D= type 6a.
Patterns E and F represent sequences obtained in this study.

Table 5.3.3b HCV GENOTYPE DISTRIBUTION IN BLOOD DONORS FROM HONG KONG

Genotype	No. positive samples	%
1	140	66.0
1a	13	6.1
1b	127	59.9
2	11	5.2
2a	8	3.8
2b	3	1.4
3a	4	1.9
6a	56	26.4
Mixed*	1	0.5
Total	212	100%

* Mixed infection with genotypes 3a and 6a

Figure 5.3.3b PHYLOGENETIC ANALYSIS OF CORE SEQUENCES AMPLIFIED FROM HONG KONG BLOOD DONORS INFECTED WITH HCV TYPE 6a



Core sequences amplified from blood donors infected with type 6a were compared with representatives of other genotypes, using the programs DNADIST and NEIGHBOR from the PHYLIP package (Felsenstein *et al.*, 1993), and are shown as an unrooted tree.

these sequences contained substitutions at positions -127 or -155 characteristic of certain type 6 group variants (Table 5.3.3a and Figure 5.3.3a). Cleavage of samples from individuals with type 6 with these enzymes produced pattern D.

RFLP analysis of type 1 samples in the core region did not reveal any of the restriction patterns associated with type 6 group variants (a10-13, s1/s5) and was successful in distinguishing between types 1a (pattern a1) and 1b (pattern a3). All type 6 samples analysed produced pattern a12s5, and these were confirmed as subtype 6a by sequence analysis of the core gene. Phylogenetic analysis of 20 type 6a core sequences revealed a high level of sequence conservation within isolates from Hong Kong, as demonstrated by a tight cluster of branches on the phylogenetic tree (Figure 5.3.3b).

The two samples producing unusual results with enzymes *Rsa* I/*Hae* III were identified as type 1b by core RFLP, and this was confirmed for one of these samples by sequencing of the core gene. All results from RFLP analysis of the 5' NCR were consistent with RFLP and sequence analysis of the core gene (Table 5.3.3a).

The remaining 139 samples were therefore typed using all 5'NCR RFLP assays. All 139 samples produced recognisable patterns of fragments with both the genotyping and subtyping RFLP assays, but unusual restriction patterns were observed for two samples (HK582 and HK620) upon digestion with *Dde* I/*Hpa* II. These patterns were similar to pattern D produced by type 6a sequences, although each contained both a 130bp fragment and an additional larger (HK582) or smaller

(HK620) band. In order to investigate the possibility that these unusual patterns represented mixed infections, virus sequences were obtained by amplification of cDNA at limiting dilution. Three different patterns were observed among single cDNA molecules amplified from HK582, including patterns A, D and a new pattern provisionally named E (Figure 5.3.3a). Single molecules from HK620 produced a combination of patterns D and a new pattern F which was similar to pattern C produced by certain type 6 group variants. Sequence analysis of the amplified 5' NCR from each of the different single molecules revealed three different virus sequences in HK582, including type 3a (which had produced pattern A by RFLP analysis) and two different sequences each containing the CA insertion at position -146 and C at position -138 characteristic of type 6a sequences. This individual therefore has a mixed infection with types 3a and 6a. The production of pattern E by these type 6a isolates in addition to pattern D can be explained by a nucleotide substitution at position -154 of C for T in one of the sequences causing the loss of an *Hpa* II cleavage site. Sequence analysis of amplified 5' NCR from the different single molecules producing restriction patterns D and F from sample HK620 revealed three distinct sequences all corresponding to type 6a. Although one of these sequences produced pattern D typical of this subtype, the nucleotide substitution of A for G at position -126 in the other two sequences presented a cleavage site for *Dde* I identical to that observed in certain type 6 group variants originally classified as NGII (Mellor *et al.,* 1996). However, despite this region of sequence identity, these sequences were classified as type 6a because of the

presence of the insertions at positions -146 and -138, which are exclusive to this subtype.

The HCV genotypes identified in this survey (Table 5.3.3b) included type 1a (n=13), 1b (n=127), 2a (n=8), 2b (n=3), 3a (n=4) and 6a (n=56) and a mixed infection with types 3a and 6a in one donor.

The reactivity of samples from donors infected with each genotype towards each of the type 1a specific antigens in the Abbott Matrix RIBA (core, NS3, and NS4) was compared (Table 5.3.3c). All samples from donors infected with type 1a were reactive against all antigens in the assay, in comparison with only 88% type 1b and 67% type 6a samples. No significant difference in the reactivity towards core or NS3 antigens was found between genotypes, but 11% type 1b sera and 32% type 6a sera were unreactive towards the NS4 antigen.

5.3.4 DISCUSSION

This survey has revealed that the majority of HCV positive blood donors from Hong Kong are infected with HCV type 1b (59.9%) or type 6a (26.4%), with the remainder infected with types 1a (6.2%), 2a (1.4%), 2b (1.4%), 3a (1.9%) or a mixed infection with types 3a and 6a (0.5%). These results are similar to those found in a previous study of 37 donors, in which type 6a was responsible for 32% infections (McOmish *et al.,* 1994), although type 3 had not been detected in this population previously. In contrast, a survey of the genotype distribution in HCV positive patients with chronic hepatitis from Hong Kong revealed type 6 infection

TABLE 5.3.3c ABBOTT MATRIX RESULTS FROM EACH GENOTYPE OF HCV INFECTED BLOOD DONORS FROM HONG KONG

Genotype	Total No. Samples	No. Samples with Matrix Reactivity (%)			
		All Antigens Positive	Core Negative	NS-3 Negative	*NS-4 Negative
1a	11	11 (100)	0 (0)	0(0)	0 (0)
1b	123	108 (88)	1 (1)	0 (0)	13 (11)
2a	8	3 (38)	0 (0)	0 (0)	5 (62)
2b	3	2 (67)	0 (0)	0 (0)	1 (33)
3a	4	2 (50)	0 (0)	0 (0)	2 (50)
6a	56	37 (66)	0 (0)	1 (2)	18 (32)

* at least 1 from two NS-4 reactions in the assay. (antigen expressed in both yeast and E.coli)

in only 14% samples (Zhang *et al.*, 1995). This difference may be explained if type 6a has only recently spread to Hong Kong, and had therefore not been present in the local population long enough to cause significant disease. Alternatively, it is also possible that type 6a causes a less severe course of disease than type 1b. The relative pathogenicity of types 4-6 has not yet been reported, but is discussed in section 5.5.

No type 6 group variants were identified amongst a subset of the blood donors using RFLP analysis of the 5'NCR or core, or sequence analysis of the core gene. This has lead to the conclusion that despite the coexistence of type 6 group variants with type 6a in other countries such as Vietnam, subtypes other than 6a were not prevalent in Hong Kong.

The discovery of two type 6a sequences in this study which contain previously unidentified nucleotide substitutions in the 5' NCR, brings into question the efficiency of cleavage with the enzymes *Dde* I/*Hpa* II for the identification of type 6 group variants. Although these sequences have new restriction patterns (E and F), these differ from those already described for type 6 variants (patterns B and C) by an increase of only 3 bp. This small difference in size is unlikely to be clearly discovered upon electrophoresis of cleaved DNA fragments, and it is likely that such variants would be misidentified as type 6 group variants. Despite this confusion between type 6 isolates, the assay was consistently accurate in the identification of type 1 samples in this population.

An important factor in the prevention of HCV transmission is the efficient detection of the virus in blood donors. Current screening assays use antigens derived from type 1 sequences, and a reduced reactivity of antibody generated towards types 2, 3 and 4 has been reported (McOmish *et al.*, 1994; Dhaliwal *et al.*, 1996), leading to the possibility that virus detection could be less effective in countries where novel genotypes are prevalent. A lower frequency of antibody reactivity towards type 1 antigens in the RIBA was also detected with type 6a samples in this study, suggesting that the type-specific reactivity reported for types 2, 3 and 4 may also apply to type 6a. The success of HCV detection within such populations may be increased in the future by the development of screening assays that use a combination of antigens from the full range of genotypes prevalent in that area.

5.4 TESTING THE 1-6 SEROTYPING ELISA ON HCV POSITIVE BLOOD DONORS IN HONG KONG

5.4.1 INTRODUCTION

A previous study has suggested that the 1-6 serotyping ELISA may be less sensitive towards antibody produced in individuals infected with type 6 (66.6%) in comparison with antibody elicited upon infection with type 1 (92.3%) or type 2 (96.9%). However, the number of samples from individuals infected with type 6 available for analysis in previous studies (n=6) has been limited. The sensitivity of

the assay to type 6, might be low because the type 6 peptides in the 1-6 ELISA were designed from a comparison of only two sequences, and might therefore not fully represent the level of amino acid variation within this genotype.

The availability of a large number of HCV positive samples from Hong Kong has allowed a more thorough investigation of the ability of the 1-6 serotyping assay in the detection of type-specific antibody in individuals infected with type 6, and the extent of variation of type 6a sequences within NS4 antigenic regions 1 and 2.

5.4.2 SAMPLES

A total of 212 HCV positive blood donor samples in which the genotype of infecting virus had been determined by RFLP analysis (Section 5.3) were tested using the 1-6 serotyping assay.

5.4.3 RESULTS

(1) SEROTYPING

Type-specific antibody was detected in 164/212 HCV positive samples, giving an overall sensitivity of 77.7% (Table 5.4.3). A difference in the sensitivity of the assay for individual HCV genotypes was noted, although the relevance of results for types 2a, 2b and 3a is questionable due to the small number of samples present in this study. The analysis of a larger number of samples from individuals infected with types 1b and 6a (n=128 and 55 respectively) revealed that the ELISA

TABLE 5.4.3 DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV POSITIVE BLOOD DONORS FROM HONG KONG USING THE 1-6 SEROTYPING ASSAY.

Genotype (RFLP)	Serotype 1	2	3	6	NTS*	NR†	Mixed	Total	Sensitivity	Concordance
1	116	-	-	1	4	19	1	141	83.7%	99.2%
a	*10*	-	-	-	*1*	*2*	-	*13*	*76.9%*	*100%*
b	*106*	-	-	*1*	*3*	*17*	*1*	*128*	*84.4%*	*99.1%*
2	-	7	-	-	1	3	-	11	63.6%	100%
a	-	*4*	-	-	*1*	*3*	-	*8*	*50.0%*	*100%*
b	-	*3*	-	-	-	-	-	*3*	*100%*	*100%*
3	-	-	2	1	-	1	-	4	75.0%	66.7%
6a	3	-	-	31	15	5	1	55	63.7%	91.4%
Mixed (6+3)	-	-	1	-	-	-	-	1	-	-
Total								212	77.7%	97.0%

* Non-type-specific reactivity (appendix 1.0)

† Non reactive

was more sensitive towards type 1b antibody (84.4%) than type 6a antibody (63.7%).

In all but five samples, the type-specific antibody detected by the assay was concordant with the genotype previously identified by RFLP analysis, giving the assay a specificity of 97.0%. Of the five samples producing discordant results, three were from individuals infected with type 6a but were identified by the serotyping assay as type 1. The other discrepant results consisted of a donor infected with type 1b, and a donor infected with type 3a both of which serotyped as type 6.

One sample confirmed as having a mixed infection with types 3a and 6a by PCR based genotyping (section 5.3) contained type-specific antibody to only to type 3a. However, a mixture of type-specific antibody was present in two other samples. In each of these samples the genotype identified by RFLP was concordant with one of the types identified by the serotyping assay.

(2) SEQUENCE ANALYSIS OF NS4

To investigate the specificity of type 6a peptides used in the assay, a total of 12 samples were amplified in the NS4 region by nested PCR and analysed by sequencing (Figure 5.4.3a). Comparison of the sequences revealed a high level of amino acid conservation within both antigenic regions 1 and 2, and a tight clustering of branches on a phylogenetic tree (Figure 5.4.3b). Amino acid substitutions were limited, with only two changes from the peptide sequence in region 1 (glutamate for lysine at position 1707 in one sample, and valine for

Figure 5.4.3a A COMPARISON OF TYPE 6a NS4 AMINO ACID SEQUENCES AMPLIFIED FROM BLOOD DONORS IN HONG KONG

```
          1680       1690       1700       1710       1720       1730       1740       1750
            ▼          ▼          ▼          ▼          ▼          ▼          ▼          ▼
                                                                                          Serotype
PT        GCVVIVGRVVLSGKPAIIPDREVLYREFDEMEECSQHLPYIEQGMMLAEQFKQKALGLLQTASRQAEVIAPAVQTNW

HK-4      .....C..IT.T....VV....I..QQ........K.I..LAE.QQI....R..V.....ASAK...ELK...HSA.
T3950     .....C...T.T....VV....I..QQ........R.I..LAE.QQI....R..V.....ASAK...ELK...HSA.

HK77             T....VV....I..QQ........R.I..LAE.QQI....R..V?....ASAK...ELK.          6
HK94           IT.T....VV....I..QQ........R.I..LAE.QQI....R..VF....ASAK...EL           6
HK97           IT.T....VV....I..QQ..?.....R.I..LAE.QQI....R..V?....ASAK...EL           6
HK99           IT.T....VV....I..QQ........RDI..LAE.QQI....R..V.....ASAK...ELK...       6
HK103          IT.?....VV....I..QQ........R.I..LAE.QQI....R..V.....ASAK...ELK....S     6
HK110       .C..IT.T....VV....I..QQ..?.....R.I..LAE.QQI....R..V...                      6
HK113     ..E..C..IT.T....VV....I..QQ........R.I..LAE.QQI....R..V.....ASAK...ELK...HSA. 6
HK114       .C..IT.T....VV....I..QQ..?.....R.I..LAE.QQI....R..V.....ASAK...             1
HK125         T.TS...IV....I..QQ........R.I..LAE.QQI....R..V.....ASAK...ELK...HSA.     6
HK150       .C..IT.T....VV....I..QQ........R.I..LAE.QQI....R..V.....ASAK...ELK.R.I.     6
HK157       .C..IT.T....IV....I..QQ........R.I..LAE.QQI....R..V.....ASAK...ELK.         6
HK185         ..IT.T....VV....I..QQ..K.....R.I..LAE.QQI....R..V.....ASAK...ELK..        6
```

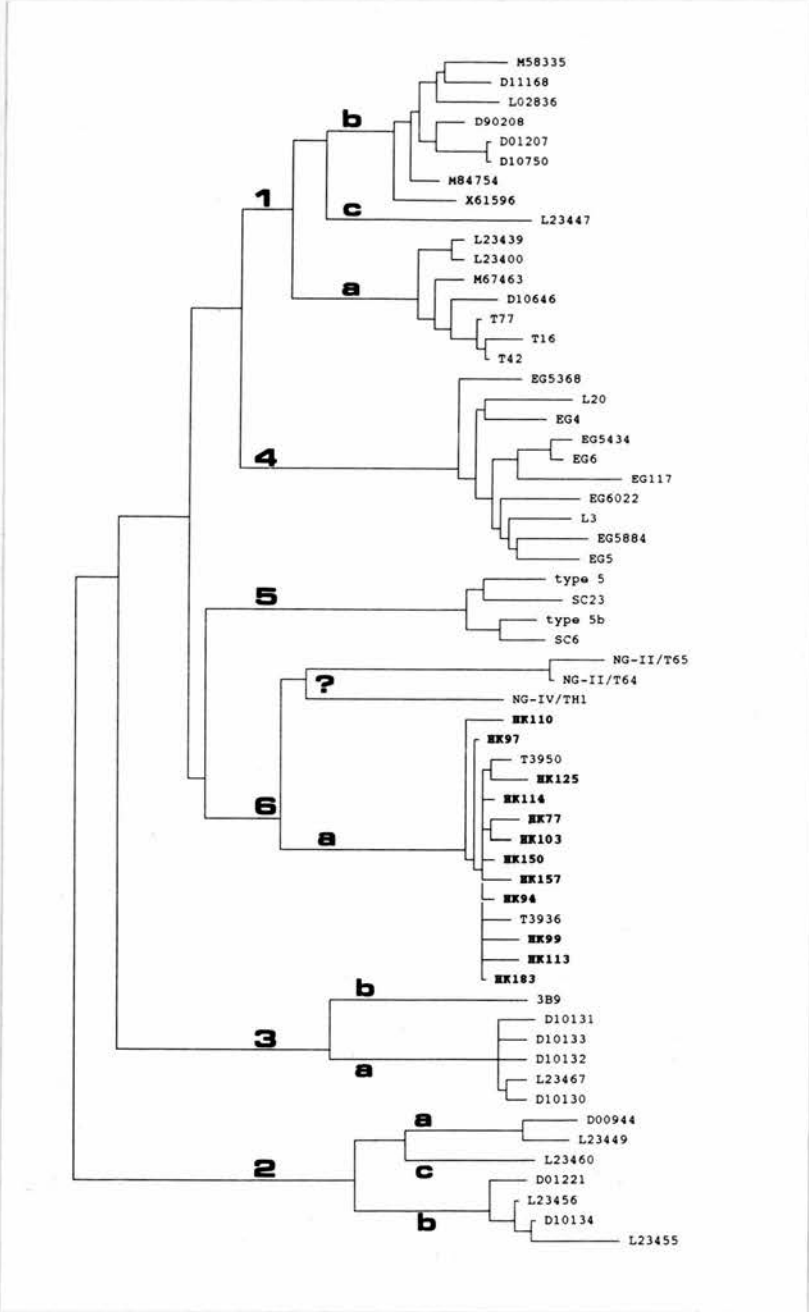
Sequences are compared with the HCV prototype sequence (HCV-PT; Choo *et al.*, 1991), and two other sequences from type 6a isolates (HK-4 and T3950).

? Sequence undetermined

"." Sequence identity to HCV-PT

NS4 antigenic regions 1 and 2 are underlined

Figure 5.4.3b PHYLOGENETIC ANALYSIS OF NS4 SEQUENCES AMPLIFIED FROM HONG KONG BLOOD DONORS INFECTED WITH HCV TYPE 6a



NS4 sequences amplified from blood donors infected with type 6a were compared with representatives of other HCV genotypes, using the programs DNADIST and NEIGHBOR in the PHYLIP package (Felsenstein *et al.*, 1993) and shown as an unrooted tree (appendix 5.3).

Sequences from Hong Kong blood donors infected with type 6a are highlighted in bold.

isoleucine at position 1694 in two sequences) and one substitution within region 2 (histidine for aspartate at position 1714 in one sample). Sequence analysis of sample HK114 confirmed the presence of HCV-6a, despite this being identified as type 1 by the serotyping assay, and moreover, the sequence obtained from this sample was identical to that of the peptides used in the 1-6 serotyping assay.

5.4.4 DISCUSSION

The results of a previous study using the 1-6 serotyping assay for the detection of type-specific antibody from samples infected with type 6 suggested that the assay may have a lower sensitivity towards type 6 (66.6%) in comparison to types 1-5 (Chapter 3, Bhattacherjee *et al.*, 1995), although the small number of samples analysed challenges the significance of this conclusion. The availability of a larger number of samples in this study enabled a more thorough investigation which confirmed the original findings, with type-specific antibody being detected in only 63.7% samples from individuals infected with type 6. In comparison, the assay was able to recognise type-specific antibody in 84.4% samples from individuals infected with type 1b, which also agreed with previous findings (83.3%).

The overall sensitivity of the assay for samples from Hong Kong blood donors was 77.7%, and antibody specificity was concordant with the genotype identified by RFLP in 97% of samples. The sensitivity of the assay in Hong Kong was lower than observed in other studies, for example in Norway (93.5%) and an

investigation of a wider range of geographical areas (87.1%), although the assay was also less effective in other regions such as Pakistan (52.3%) and Egypt (72.5%) (Chapter 3). The reduced sensitivity of the serotyping ELISA in Pakistan may have resulted from a higher level of amino acid sequence variation within type 3 than accounted for by the peptides used in the assay. Indeed, a large number of type 3 subtypes (a-f) have now been identified (Tokita *et al.,* 1994a). The presence of antigenic variation within type 6 in Hong Kong was investigated by the sequence analysis of NS4, revealing that type 6 isolates were well conserved within this region, and that the type 6 peptides used in the assay should be sufficiently specific. A high level of sequence conservation within type 6a has also been described in the core region (section 5.3 and Zhang *et al.*, 1995, Mellor *et al.*, 1995). The relatively high number of type 6 samples producing NTS results with the 1-6 serotyping assay suggests that another explanation for the low sensitivity towards type 6 may be the event of cross reactivity with heterologous peptides present in competing solutions. It has been noticed previously that region 1 peptides for types 4-6 are similar to those for type 1, and therefore the successful detection of type-specific antibody in samples infected with these genotypes may be more reliant on antibody generated to region 2. It is also possible that region 1 is more antigenic in individuals infected with type 6 isolates than for other genotypes, so that the majority of antibody in type 6 samples cross reacts with region 1 competing peptides and gives a NTS result with the assay. Epitope mapping of type 6 could confirm this hypothesis.

5.5 DETECTION OF TYPE-SPECIFIC ANTIBODY IN SAMPLES FROM INDIVIDUALS INFECTED WITH TYPE 6 GROUP VARIANTS

5.5.1 INTRODUCTION

Sequence analysis of HCV type 6 group variants has revealed a higher level of sequence similarity with genotype 6a than any of the other major genotypes (Mellor *et al.,* 1995). However, a number of these isolates show identity to type 1 sequences in the 5' NCR, which has led to the misidentification of these viruses using existing typing assays such as RFLP analysis of the 5' NCR (Davidson *et al.,* 1995) and Inno-LiPA (Stuyver *et al.,* 1996b). This problem has been overcome to a certain extent by use of a core RFLP assay (Mellor *et al.,* 1996), as sufficient sequence variation is present in the core region of type 6 isolates to enable this genotype to be distinguished from type 1. As the serotyping ELISA is often a cheaper alternative to genotyping methods involving PCR, an accurate assay which is able to identify these regional variants would be valuable in poorer countries.

The ability of the 1-6 serotyping assay to detect antibody generated during infection with novel type 6 group variants was investigated, and the NS4 amino acid sequences of such isolates compared with the type 6a peptides used in the ELISA.

The samples from individuals infected with type 6 group variants, classified as NGI (also classified as type 8a/b; n=1), NGII (type 7a/b; n=5) and NGIV (type

9a; n=4), were obtained from Thailand blood donors, as described in previous studies (Mellor *et al.,* 1995; Mellor *et al.,* 1996).

5.5.2 RESULTS

A low level of reactivity towards type 6 peptides was observed in the sample from an individual infected with NGI, and in 2 out of 5 samples from individuals infected with NGII (Table 5.5.2). However, other samples from individuals infected with NGII displayed reactivity towards both type 1 and 6 peptides, to type 3 peptides alone or non-type-specific reactivity. Type-specific antibody was not detected in any of the samples from individuals infected with NGIV.

NS4 regions were amplified and sequenced from isolates corresponding to NGII and NGIV, revealing identity with a significant number of amino acids from the antigenic region 1 type 6a peptide used in the serotyping ELISA, for example, the two glutamine residues at positions 1703 and 1704 (Figure 5.5.2). In antigenic region 2, sequences from NGII differed from type 6a by only one amino acid substitution of alanine for valine at position 1719, whereas NGIV sequences had four amino acid substitutions between positions 1718-1724 (Figure 5.5.2).

5.5.3 DISCUSSION

Although the recently identified type 6 group variants in South East Asia have been shown to have a closer genetic relationship to type 6a than to other

Table 5.5.2 DETECTION OF TYPE-SPECIFIC ANTIBODY IN HCV POSITIVE BLOOD DONORS FROM THAILAND INFECTED WITH TYPE 6 GROUP VARIANTS

Sample	Provisional name*	Classification†	Serotype
EUTH 49	NG I	(type 8)‡	6 (weak)
EUTH 7	NG II	(type 7c)	1+6 (weak)
EUTH 28	"		6
EUTH 36	"		3
EUTH 61	"		NTS¶
EUTH 89	"		6
EUTH 1	NG IV	(type 9c)	NTS
EUTH 21	"		NTS
EUTH 22	"		NR
EUTH 39	"		NTS

* as described by Mellor *et al.*, 1996

† Previous classification in parenthesis (Tokita *et al.*, 1994, 1995)

‡ Groups with, but distinct from, types 8a and 8b found in Vietnam upon phylogenetic analysis

¶ Non-type-specific reactivity

Figure 5.5.2 AMINO ACID SEQUENCES OF TYPE 6 GROUP VARIANTS IN NS4 ANTIGENIC REGIONS 1 AND 2.

```
          1690        1700          1710        1720
           |           |             |           |
HCV-1a     G KPAIIPDREVLYREFDEM E ECSQHLPYIEQGMMLAEQF KQ
HCV-6a     . ...VV....I..QQ.... . ...R.I..LAE.QQI.... R.
HCV-NGII   . ..VPM.......QQY... . ...R.I..LVE.QQI.... ..
HCV-NGIV   . Q.VL......S.QQ.... . ...R.I..LPAEQAI.... ..
```

major genotypes, the level of similarity observed overlaps the boundaries previously identified between subtypes of the same virus type and this has produced confusion over their classification. Analysis of samples from individuals infected with types NGI, NGII and NGIV by the 1-6 serotyping assay, revealed the presence of type 6 specific antibody in some samples from individuals infected with NGII. This observation is partially explained by sequence analysis of NS4 for both NGII and NGIV, since the extent of amino acid variation observed between these variants and the type 6a peptides used in the assay was greater for the NGIV sequence than for the NGII sequence. In addition, many of the amino acid substitutions in NGII were to residues of similar size or charge compared with those present in the type 6a peptides. Substitutions in the NGIV sequences were more likely to alter the structure or hydrophobicity of the antigen, for example, alanine (small, hydrophobic) for proline (hydrophilic and prone to influence protein architecture) at position 1719 and glutamine (hydrophilic, uncharged) for alanine (hydrophobic) at position 1723.

For the 1-6 serotyping assay to be able to successfully identify the type 6 group variants, additional peptides would have to be incorporated into the assay. Although this is theoretically possible, the relatively confined geographical distribution of these isolates fails to justify such measures in areas such as the U.S.A., Europe and Japan, where these genotypes are unlikely to be encountered in the indigenous population. In populations where the type 6 group variants are more common, an single additional well containing antigens representing all of

these variants together may be beneficial, and this would certainly be a more practical solution for the addition of these variants to the existing assay. Such an assay may also prove important in Western populations in the future, as world travel continues to increase, creating a risk that these isolates may spread to different geographical areas. This has already been the case with types 5 and 6a now detected in Canada (Bernier *et al.,* 1996) and type 6a in Australia (Dr L. Moaven, *pers. comm*).

5.6 CLINICAL FEATURES ASSOCIATED WITH INFECTION WITH HCV TYPE 6a

5.6.1 INTRODUCTION

The epidemiology and clinical features HCV genotypes 1, 2 and 3 have been extensively studied in recent years, revealing a number of differences between these virus genotypes. For example, infection with type 3 has been associated with younger patients who are more likely to have IVDA as a risk factor in Scotland (McOmish *et al.,* 1993), France (Pawlotsky *et al.,* 1995) and in the U.S.A. (Lau *et al.,* 1996), while a higher proportion of infections with type 1b have been acquired through blood transfusion. Other clinical features such as the level of ALT have are similar between the types 1-3, but may be lower for type 4 (Lau *et al.,* 1996). In contrast, there is no difference between any of the six major genotypes in the level of viraemia produced upon infection. The ability of HCV type 1 to cause a more severe disease than types 2 or 3 is currently a matter of debate, with numerous reports both in favour (Kobayashi *et al.,* 1996; Prati *et al.,* 1996; Booth *et al.,* 1995; Dusheiko *et al.,* 1994) and to the contrary (Benvegnu *et al.,* 1997; Zeuzem *et al.,* 1996; Zhou *et al.,* 1996).

Unlike genotypes 1-3, very few studies have been performed on the clinical features associated with types 4-6, and such investigations may be important in the identification of either routes of transmission, disease progression or the response to treatment. As the first step in such a study for infection with type 6, information

such as sex, age, ALT level and risk factors for infection was compared for a total of 210 Hong Kong blood donors infected with different HCV genotypes.

5.6.2 RESULTS

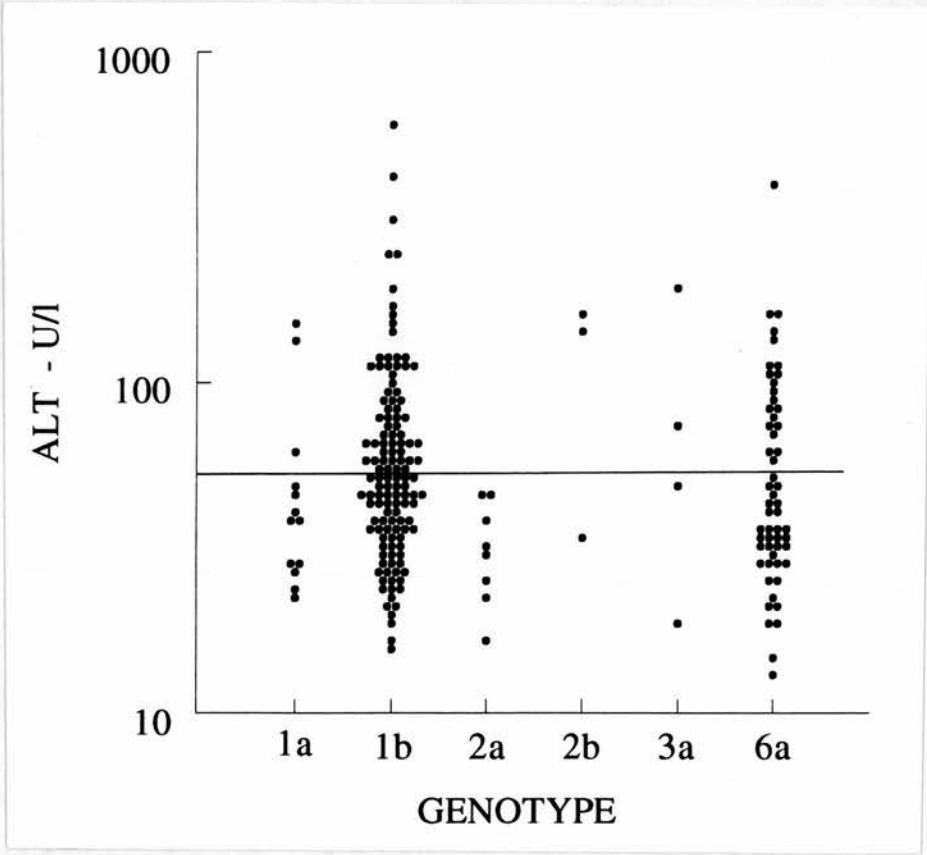
More males than females were infected with all genotypes, but this bias was even greater for type 6a infections (5:1), than for type 1b infections (3:1). In contrast, the mean age for infections with types 1b and 6a was similar at 33 and 34 years respectively, and the age of infected donors in Hong Kong ranged between 16 and 59 years (Figure 5.6.2a), and was also similar for all genotypes. Median ALT values (Figure 5.6.2b) were in the normal range (7-53U/l) for all genotypes except type 2b, although only three samples were infected with this genotype. ALT values in donors infected with type 6a were lower than for infection with type 1b, but this difference was not statistically significant by the Spearmans rank correlation ($p=0.094$).

Risk factors were available for 43 samples (Table 5.6.2), and included blood transfusion prior to 1989, IVDA, tattooing and acupuncture. While 66% of donors infected with type 6a reported IVDA as a risk factor, this was true for only 7% donors infected with type 1b. Similarly, only 33% of individuals infected with type 6a had received a blood transfusion prior to 1989 in comparison to 83% of donors infected with type 1b.

Table 5.6.2 HCV GENOTYPE DISTRIBUTION AND CLINICAL FEATURES IN HONG KONG BLOOD DONORS

Genotype	No. Positive		Donor Sex (%)		Mean Age	Risk Factors					
	(%)		Male	Female	(Range)	Total Samples Available	Blood Trans.	IVDA	Tattoo	Acupuncture	None
HCV-1a	13	(6.2)	10 (77)	3 (23)	35 (16-47)	2	1	1	0	0	0
HCV-1b	124	(58.8)	93 (75)	31 (25)	33 (17-59)	27	15	2	5	1	9
HCV-1	*137*	*(65.0)*	*103 (76)*	*34 (24)*	*34 (16-59)*	*29*	*16*	*3*	*5*	*1*	*9*
HCV-2a	8	(3.8)	2 (25)	6 (75)	31 (25-48)	2	0	0	2	0	0
HCV-2b	3	(1.4)	3 (100)	0 (0)	37 (34-39)	0	0	0	0	0	0
HCV-2	*11*	*(5.2)*	*5 (45)*	*6 (55)*	*34 (25-48)*	*2*	*0*	*0*	*2*	*0*	*0*
HCV-3a	4	(1.9)	4 (100)	0 (0)	35 (24-42)	3	1	2	1	0	0
HCV-6a	57	(27.0)	48 (84)	10 (16)	34 (16-52)	9	3	6	4	0	0
MIXED	1	(0.5)	1 (100)	0 (0)	n/a	1	0	1	1	0	0

Figure 5.6.2b COMPARISON OF ALT VALUES OF DONORS INFECTED WITH DIFFERENT HCV GENOTYPES IN HONG KONG



Line indicates upper normal ALT range.

5.6.3 DISCUSSION

Donor populations generally contain a preponderance of male donors, and a larger proportion of male blood donors infected with type 6a in comparison to 1b has also been reported previously in the U.S.A. for other genotypes such as types 3 and 4 (Lau *et al.,* 1996).

The association of infection with HCV type 6a in this study with the risk factor of IVDA is not in agreement with a previous analysis in which a total of 8 patients infected with type 6a were thought to have contracted the disease through blood transfusion (n=7) or occupational exposure (n=1) (Zhang *et al.,* 1995). Infection with type 3 has also been linked with IVDA in previous studies, however, this finding was also connected with a lower age of acquisition, whereas in this study there was no difference in the age distribution between different genotypes.

The median ALT level in donors infected with type 6a was slightly lower than that observed in type 1b infections, although this was not statistically significant. The majority of studies have reported no difference in ALT levels between different genotypes (Dusheiko *et al.,* 1994; Smith *et al.,* 1996; Dhaliwal *et al.,* 1996), however, a higher ALT level with type 3 has been reported by some groups (Preston *et al.,* 1995; McOmish *et al.,* 1993), although this is not necessarily reflective of more severe disease.

The restricted diversity of type 6a is consistent with the recent spread of this genotype into Hong Kong. This hypothesis would also explain its specific association with drug use as this has become the major route of HCV transmission

in most countries since screening blood donors for HCV. Finally, the under-representation of type 6a in hepatitis patients (Zhang *et al.*, 1995) also suggests recent entry of this genotype to the Hong Kong population, as clinically significant disease may take 20-30 years to become recognised. Clearly a valuable further investigation would be a specific comparison of genotype distribution in stratified age groups. As a possible analogy, the spread of type 6a to Australia was initially associated with the immigration of Vietnamese nationals (Dr. L. Moaven. Pers. Comm.), and has since become specifically associated with drug abuse in major cities such as Melbourne and Sydney.

CHAPTER 6

6 SEQUENCE ANALYSIS OF HCV VARIANTS PRODUCING DISCREPANT RESULTS WITH TWO DIFFERENT TYPING ASSAYS

6.1 GENERAL INTRODUCTION

Two of the most widely used commercial typing assays are the 1-6 serotyping ELISA described in this work (HC02 - Murex Biotech) and a line probe assay (InnoLiPA 2.0, Innogenetics, Belgium). Both assays have various advantages and disadvantages. The line probe assay is able to distinguish between subtypes 1a and 1b, and 2a/c and 2b and other major genotypes 3-6, and since it involves the direct detection of virus RNA it can be used in immunocompromised patients. On the other hand, this method involves the amplification of HCV RNA by PCR, which is expensive, time consuming and may require specialised equipment that is not routinely available in a clinical laboratory. The serotyping assay can be performed directly on the serum from an infected individual within 3 hours using non-specialised equipment, but is unable to distinguish virus subtypes or be used in immunocompromised patients.

The specificity of both methods is high, with the 1-6 serotyping assay showing a concordance (see appendix 1.0) of 97% (Bhattacherjee *et al.,* 1995), 98% (Bell *et al.,* 1996) and 100% (Navas *et al.,* 1997) with RFLP, and the InnoLiPA showing a concordance of 98.2% (Lau *et al.,* 1996). The InnoLiPA and

serotyping assays have also been reported as being 98.5% concordant with each other (Lau *et al.,* 1996).

Although the specificity of both the InnoLiPA genotyping and 1-6 serotyping assays are high, a small number of samples have been shown to produce discrepant results between the two assays. As the InnoLiPA is based on the reverse hybridisation of type-specific probes to amplified 5'NCR sequences, the specificity of this assay relies on the high level of sequence conservation of the 5'NCR within individual HCV genotypes. One of the major strengths of this assay, which allows the discrimination of a number of subtypes as well as genotypes, is the sensitivity of the probes towards even single nucleotide differences (Stuyver *et al.,* 1996b; Stuyver *et al.,* 1993b). However, this level of sensitivity may also provide an explanation for discrepant results produced by this assay, since variants with unusual nucleotide substitutions in the 5' NCR might hybridise, or fail to hybridise, to probes specific for a particular genotype. Similarly, antigenic variation within the region of NS4 corresponding to the peptides used in the serotyping assay could lead to the binding of antibody towards heterologous type peptides, and produce an inaccurate serotyping result. Although the levels of genetic variation within individual genotypes has been shown to be low for both the 5' NCR (Smith *et al.,* 1995b; Simmonds *et al.,* 1993b), and NS4 (Bhattacherjee *et al.,* 1995), reflected in the high specificity of both these assays, an increasing number of unusual HCV variants are being reported (Tokita *et al.,* 1995; Mellor *et al.,* 1995; Tokita *et al.,*

1994; Stuyver *et al.,* 1996; Smith *et al.,* 1995) which may test the limits of these assays.

The successful identification of HCV genotypes using methods based on subgenomic regions is also dependent on the assumption that such regions are representative of the genome as a whole. Recombination between different HCV genotypes could lead to the discrepant typing of a sample if the two assays were based on different regions of the genome. Although recombination has been observed in other RNA viruses, there are few reports describing this event in HCV (Yun *et al.,* 1996; Kato *et al.,* 1992), and the possibility of artifactual recombination during PCR (Meyerhans *et al.,* 1990), PCR contamination or a mixed infection have not been ruled out in these studies. In contrast, the great majority of groups have reported that sequence relationships of subgenomic regions consistently reflect those of complete genomes (Stuyver *et al.,* 1994; Simmonds *et al.,* 1994; Smith *et al.,* 1995; Ohba *et al.,* 1995).

Finally, discordant results obtained with the two assays could be explained by the difference in detection of virus RNA (InnoLiPA) and circulating antibody (serotyping ELISA) in individuals infected with more than one HCV genotype. If an individual infected with HCV is reinfected with a different genotype, it is likely that the genotype of virus identified directly by PCR based methods such as InnoLiPA would differ from the serotype of circulating antibody during the "window period" for the superinfecting virus. Similarly, the "reactivation" of

different genotypes within a mixed infection is often followed by a corresponding change in the serotype of antibody detected (Jarvis *et al.,* 1994).

A number of discrepant samples were obtained from two studies of a group of haemophiliacs and a group of patients with chronic hepatitis C. Virus sequences in these samples were investigated by sequencing in the 5' NCR, core and NS4 regions in order to confirm the virus genotype and identify the possible reasons for the differences in typing results.

6.2 SAMPLES

Haemophiliacs

A total of 11 samples producing discrepant results with InnoLiPA (second generation assay) and 1-6 serotyping assays were obtained from a study of 78 HCV PCR positive haemophiliacs from Frankfurt in Germany. For 8 samples the genotype identified in the 5' NCR (InnoLiPA) was type 3a, while type 1 specific antibody was detected by the serotyping assay (Table 6.2). Two samples (GD10 and GD11) were type 2b according to InnoLiPA and type 1 by serotyping, and sample GD6 was the opposite, with type 1b by InnoLiPA and type 2 by serotyping. The remaining sample (GD1) was type 3a in the 5' NCR while a mixed infection with types 1 and 2 were detected by the serotyping assay.

Patients with chronic hepatitis C

From a study involving 88 patients with chronic hepatitis C in France, 4 samples produced discrepant results between the serotyping and InnoLiPA assays.

Table 6.2 Identification of Genotypes in Discrepant Samples Using Different Typing Assays and Sequencing.

Sample*	Typing Results		Type-Specific PCR			Sequencing results		
	Inno-LiPA II	Murex 1-6	T1	T2	T3	5'NCR	Core	NS4
GD1	3a	1+2	-		+	3a	-	3a
GD2	3a	1	+w		+	3a	3a	3a
GD3	3a	1	-		+	3a	3a	3a
GD4	3a	1	-		+	3a	3a	3a
GD5	3a	1	-		+	3a	-	3a
GD6	1b	2	+	-		1b	1b	1b
GD7	3a	1	-		+	3a	-	3a
GD8	3a	1	+w		+	3a	3a	3a
GD9	3a	1	-		-	3a	-	-
GD10	2b	1	-	+		2b	2b	2b
GD11	2b	1	-	+		2b	2b	-
	Inno-LiPA I							
FD1	1b	4				1b	1b	1b
FD2	4-5	1				5	5	5
FD3	3a	1				3a	3a	3a
FD4	3a	1				3a	3a	3a

"w" = weak result * GD= German discrepant (haemophiliacs); FD= French discrepant (chronic hepatitis patients)

Two of these samples (FD3 and FD4) were HCV-3a by InnoLiPA (1st generation assay) but type 1 by serotyping (Table 6.2). Sample FD1 was identified as type 1b in the 5' NCR while the serotyping ELISA detected type 4 specific antibody, and sample FD2 was type 4 or 5 by InnoLiPA, but type 1 by serotyping.

Genotyping and serotyping was carried out in the country of origin, by Dr. A. Berger (Institut für Medizinische Virologie der J.W. Goethe-Universität Frankfurt, Germany) and Dr. J-M Pawlotsky (Department of Bacteriology and Virologie, Hôpital Henri Mondor, Université de Paris XII, Crétiel, France).

6.3 RESULTS

6.3.1 HCV POSITIVE HAEMOPHILIACS

RNA sequences from all 11 samples were amplified by PCR in the 5' NCR, and for the majority of samples, also in the core and NS4 regions. The HCV genotype identified by sequence analysis of the 5' NCR was concordant with that detected by the InnoLiPA assay in all 11 samples (Figure 6.3a), and in each case was confirmed by phylogenetic analysis of sequences amplified from either the core or NS4 regions.

Since haemophiliacs have been multiply exposed to virus through infected batches of factor concentrates derived from many blood donors, the presence of a dual infection with different HCV genotypes was investigated using PCR primers

specific for NS4 of types 1, 2 and 3. In all but two samples, a positive PCR result was observed only with the primers corresponding to the genotype identified by InnoLiPA (Table 6.2). In samples GD2 and GD8, NS4 sequences were amplified weakly with type 1 primers and more strongly with type 3 specific primers. Multiple clones from each PCR product were analysed by sequencing, revealing the presence of type 3a in all cases irrespective of the primers used for the PCR. There was little amino acid variation within NS4 antigenic regions 1 and 2 in these samples, with isolated amino acid substitutions to residues of similar size or charge as those present in the corresponding peptide used in the serotyping assay (Figure 6.3b)

6.3.2 PATIENTS WITH CHRONIC HEPATITIS C

All four discrepant samples from French patients with chronic hepatitis C were successfully amplified by PCR in the 5' NCR, core and NS4 regions. Sequence analysis in all three regions confirmed the genotype identified by the first generation InnoLiPA, with sample FD2 recognised more specifically as type 5 (Table 6.2).

Amino acid substitutions in NS4 were more frequent than in the haemophiliac samples, and occurred in both antigenic regions 1 and 2. In addition, the substitutions in sample FD1 produced a sequence with similarities to the type 4 peptides used in the serotyping assay (Figure 6.3b).

Figure 6.3a COMPARISON OF 5'NCR NUCLEOTIDE SEQUENCES OBTAINED FROM THE DISCREPANT SAMPLES WITH SEQUENCES SPECIFIC FOR EACH GENOTYPE

"." sequence identity to HCV-PT.

Discrepant samples are highlighted in bold.

Nucleotide positions are labelled above HCV-PT.

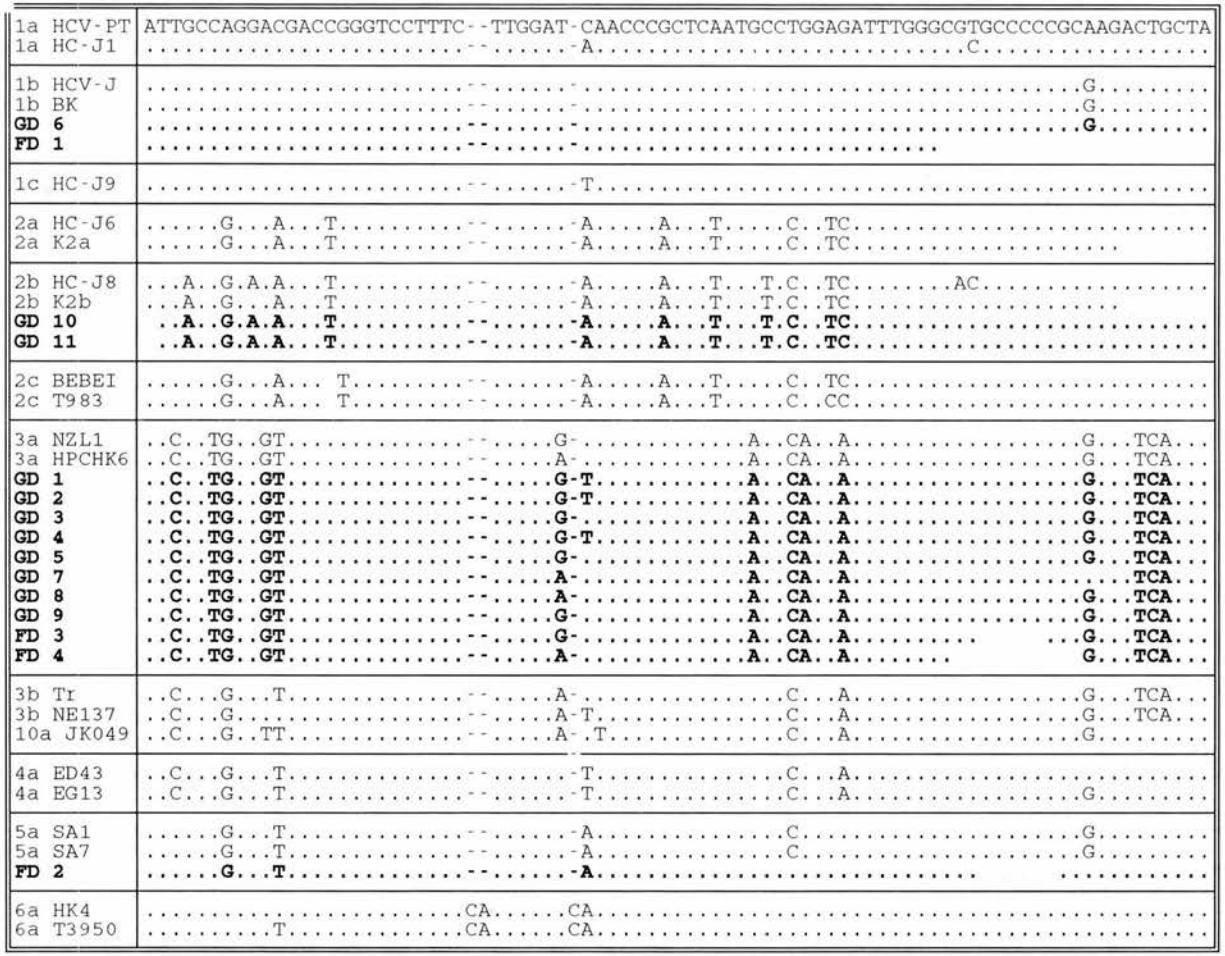
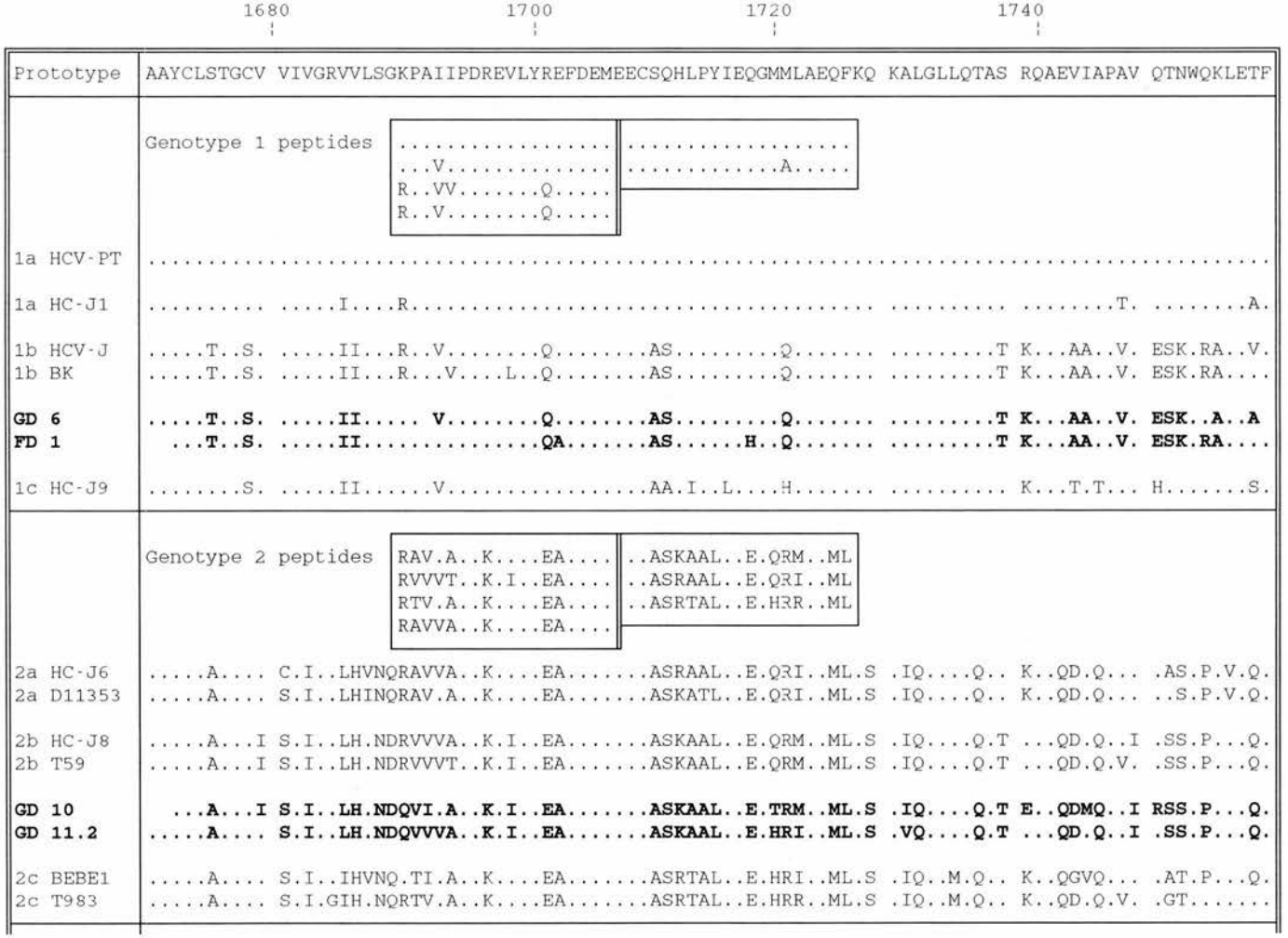

1a HCV-PT ATTGCCAGGACGACCGGGTCCTTTC--TTGGAT-CAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGACTGCTA
1a HC-J1 ..........--.....-A.....C.....
1b HCV-J ..........--.....-.....G.....
1b BK ..........--.....-.....G.....
GD 6 ..........--.....-.....G.....
FD 1 ..........--.....-.....
1c HC-J9 ..........--.....-T.....
2a HC-J6 .....G...A...T.....--.....-A.....A...T.....C..TC.....
2a K2a .....G...A...T.....--.....-A.....A...T.....C..TC.....
2b HC-J8 ...A..G.A.A...T.....--.....-A.....A...T...T.C..TC.....AC.....
2b K2b ...A..G...A...T.....--.....-A.....A...T...T.C..TC.....
GD 10 ..A..G.A.A...T.....--.....-A.....A...T...T.C..TC.....
GD 11 ..A..G.A.A...T.....--.....-A.....A...T...T.C..TC.....
2c BEBEI .....G...A... T.....--.....-A.....A...T.....C..TC.....
2c T983 .....G...A... T.....--.....-A.....A...T.....C..CC.....
3a NZL1 ..C..TG..GT.....--.....G-.....A..CA..A.....G...TCA...
3a HPCHK6 ..C..TG..GT.....--.....A-.....A..CA..A.....G...TCA...
GD 1 ..C..TG..GT.....--.....G-T.....A..CA..A.....G...TCA...
GD 2 ..C..TG..GT.....--.....G-T.....A..CA..A.....G...TCA...
GD 3 ..C..TG..GT.....--.....G-.....A..CA..A.....G...TCA...
GD 4 ..C..TG..GT.....--.....G-T.....A..CA..A.....G...TCA...
GD 5 ..C..TG..GT.....--.....G-.....A..CA..A.....G...TCA...
GD 7 ..C..TG..GT.....--.....A-.....A..CA..A.....TCA...
GD 8 ..C..TG..GT.....--.....A-.....A..CA..A.....G...TCA...
GD 9 ..C..TG..GT.....--.....G-.....A..CA..A.....G...TCA...
FD 3 ..C..TG..GT.....--.....G-.....A..CA..A.....G...TCA...
FD 4 ..C..TG..GT.....--.....A-.....A..CA..A.....G...TCA...
3b Tr ..C...G...T.....--.....A-.....C...A.....G...TCA...
3b NE137 ..C...G.....--.....A-T.....C...A.....G...TCA...
10a JK049 ..C...G..TT.....--.....A-.T.....C...A.....G.....
4a ED43 ..C...G...T.....--.....-T.....C...A.....
4a EG13 ..C...G...T.....--.....-T.....C...A.....G.....
5a SA1 .....G...T.....--.....-A.....C.....G.....
5a SA7 .....G...T.....--.....-A.....C.....G.....
FD 2 .....G...T.....--.....-A.....
6a HK4 .....CA.....CA.....
6a T3950 .....T.....CA.....CA.....

Figure 6.3b Comparison of NS4 amino acid sequences obtained from the discrepant samples with those of peptides used in the serotyping assay.



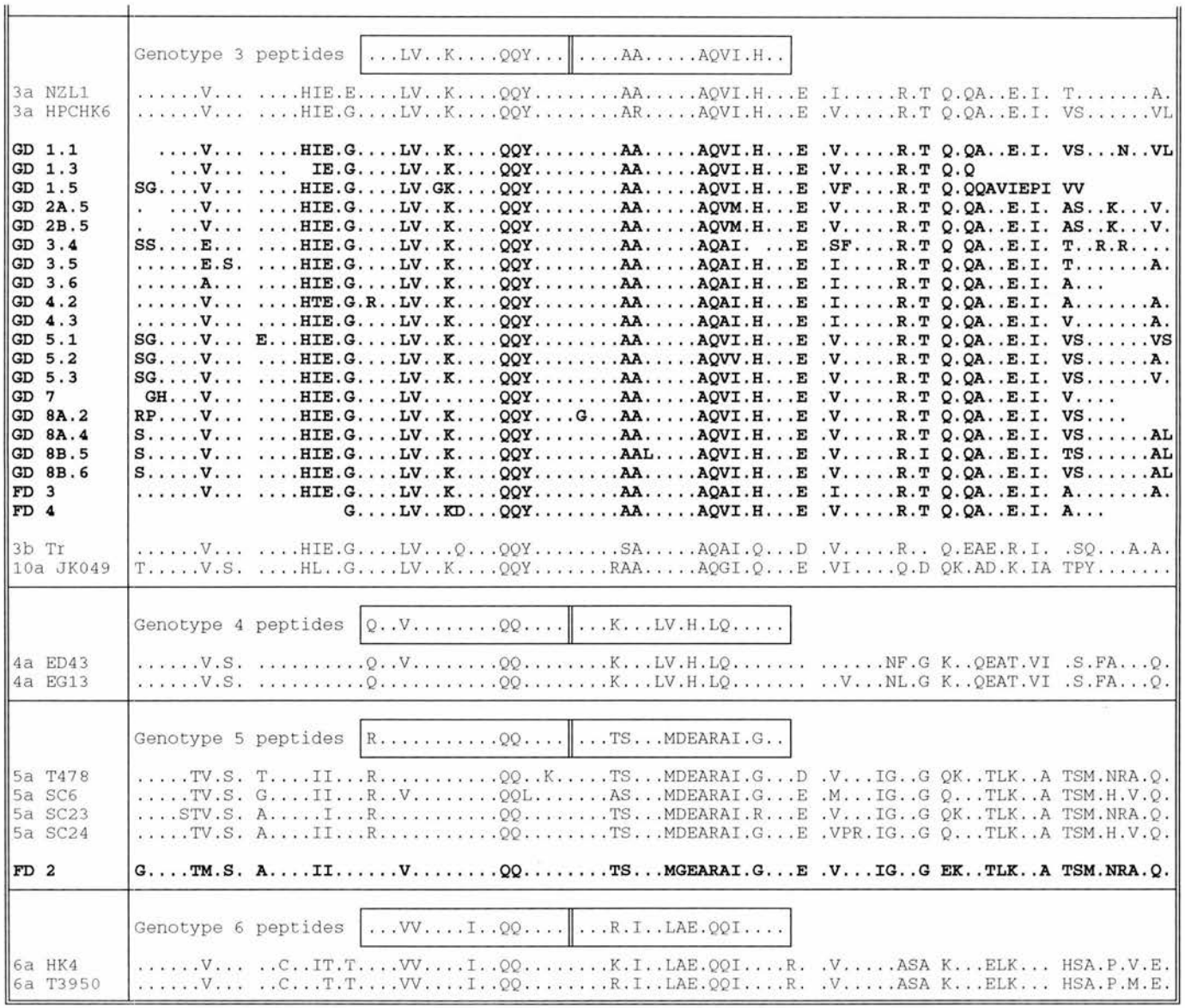

Genotype 3 peptides
...LV..K....QQY...
....AA.....AQVI.H..
3a NZL1 .....V... ....HIE.E....LV..K....QQY........AA.....AQVI.H...E .I.....R.T Q.QA..E.I. T.......A.
3a HPCHK6 .....V... ....HIE.G....LV..K....QQY........AR.....AQVI.H...E .V.....R.T Q.QA..E.I. VS......VL
GD 1.1 ....V... ....HIE.G....LV..K....QQY........AA.....AQVI.H...E .V.....R.T Q.QA..E.I. VS...N..VL
GD 1.3 ...V... ... IE.G....LV..K....QQY........AA.....AQVI.H...E .V.....R.T Q.Q
GD 1.5 SG....V... ....HIE.G....LV.GK....QQY........AA.....AQVI.H...E .VF....R.T Q.QQAVIEPI VV
GD 2A.5 . ...V... ....HIE.G....LV..K....QQY........AA.....AQVM.H...E .V.....R.T Q.QA..E.I. AS..K...V.
GD 2B.5 . ...V... ....HIE.G....LV..K....QQY........AA.....AQVM.H...E .V.....R.T Q.QA..E.I. AS..K...V.
GD 3.4 SS....E... ....HIE.G....LV..K....QQY........AA.....AQAI. ...E .SF....R.T Q QA..E.I. T..R.R....
GD 3.5 ......E.S. ....HIE.G....LV..K....QQY........AA.....AQAI.H...E .I.....R.T Q.QA..E.I. T.......A.
GD 3.6 ......A... ....HIE.G....LV..K....QQY........AA.....AQAI.H...E .I.....R.T Q.QA..E.I. A...
GD 4.2 ......V... ....HTE.G.R..LV..K....QQY........AA.....AQAI.H...E .I.....R.T Q.QA..E.I. A.......A.
GD 4.3 ......V... ....HIE.G....LV..K....QQY........AA.....AQAI.H...E .I.....R.T Q.QA..E.I. V.......A.
GD 5.1 SG....V... E...HIE.G....LV..K....QQY........AA.....AQVI.H...E .V.....R.T Q.QA..E.I. VS......VS
GD 5.2 SG....V... ....HIE.G....LV..K....QQY........AA.....AQVV.H...E .V.....R.T Q.QA..E.I. VS......A.
GD 5.3 SG....V... ....HIE.G....LV..K....QQY........AA.....AQVI.H...E .V.....R.T Q.QA..E.I. VS......V.
GD 7 GH...V... ....HIE.G....LV.......QQY........AA.....AQVI.H...E .V.....R.T Q.QA..E.I. V....
GD 8A.2 RP....V... ....HIE.G....LV..K....QQY....G...AA.....AQVI.H...E .V.....R.T Q.QA..E.I. VS....
GD 8A.4 S.....V... ....HIE.G....LV..K....QQY........AA.....AQVI.H...E .V.....R.T Q.QA..E.I. VS......AL
GD 8B.5 S.....V... ....HIE.G....LV..K....QQY........AAL....AQVI.H...E .V.....R.I Q.QA..E.I. TS......AL
GD 8B.6 S.....V... ....HIE.G....LV..K....QQY........AA.....AQVI.H...E .V.....R.T Q.QA..E.I. VS......AL
FD 3 ......V... ....HIE.G....LV..K....QQY........AA.....AQAI.H...E .I.....R.T Q.QA..E.I. A.......A.
FD 4 G....LV..KD...QQY........AA.....AQVI.H...E .V.....R.T Q.QA..E.I. A...
3b Tr ......V... ....HIE.G....LV...Q...QQY........SA.....AQAI.Q...D .V.....R.. Q.EAE.R.I. .SQ...A.A.
10a JK049 T.....V.S. ....HL..G....LV..K....QQY.......RAA.....AQGI.Q...E .VI....Q.D QK.AD.K.IA TPY.....
Genotype 4 peptides
Q..V........QQ....
...K...LV.H.LQ.....
4a ED43 ......V.S. ..........Q..V........QQ........K...LV.H.LQ..... ......NF.G K..QEAT.VI .S.FA...Q.
4a EG13 ......V.S. ..........Q...........QQ........K...LV.H.LQ..... ..V...NL.G K..QEAT.VI .S.FA...Q.
Genotype 5 peptides
R..........QQ....
...TS...MDEARAI.G..
5a T478 .....TV.S. T....II...R...........QQ..K.....TS...MDEARAI.G...D .V...IG..G QK..TLK..A TSM.NRA.Q.
5a SC6 .....TV.S. G....II...R..V........QQL.......AS...MDEARAI.G...E .M...IG..G Q...TLK..A TSM.H.V.Q.
5a SC23 ....STV.S. A.....I...R...........QQ........TS...MDEARAI.R...E .V...IG..G QK..TLK..A TSM.NRA.Q.
5a SC24 .....TV.S. A....II...R...........QQ........TS...MDEARAI.G...E .VPR.IG..G Q...TLK..A TSM.H.V.Q.
FD 2 G....TM.S. A....II......V........QQ........TS...MGEARAI.G...E .V...IG..G EK..TLK..A TSM.NRA.Q.
Genotype 6 peptides
...VV....I..QQ....
...R.I..LAE.QQI....
6a HK4 ......V... ..C..IT.T....VV....I..QQ........K.I..LAE.QQI....R. .V.....ASA K...ELK... HSA.P.V.E.
6a T3950 ......V... ..C...T.T....VV....I..QQ........R.I..LAE.QQI....R. .V.....ASA K...ELK... HSA.P.M.E.

Figure 6.3b COMPARISON OF NS4 AMINO ACID SEQUENCES OBTAINED FROM DISCREPANT SAMPLES WITH THOSE OF PEPTIDES USED IN THE SEROTYPING ASSAY

"." indicates sequence identity to HCV-PT.

Sequences of the discrepant samples are highlighted in bold.

Samples identified with a decimal point represent the reference number of the clone, for example sample GD 3.6 represents clone number 6 from sample GD3.

Amino acid sequences of type-specific peptides used in the serotyping assay are in boxes.

GD2A, GD8A; Sample GD2, GD8 amplified with type 1 specific primers.
GD2B, GD8B; Sample GD2, GD8 amplified with type 3 specific primers.

6.4 DISCUSSION

Recombination

As the two assays are based on subgenomic regions which are at opposite ends of the virus RNA, a recombinant virus with a cross over point lying between the 5' NCR and NS4 would produce different results by InnoLiPA and serotyping. In this study however, the same genotype was identified by sequencing in the 5' NCR, core and NS4 regions, providing no evidence for such an event.

Multiple infection

Discrepant results were more common in the multiply exposed haemophiliac patient group (14.1%) than in the chronic hepatitis patients (4.5%) in whom multiple exposure would be expected to be less common. In all samples the genotype identified by InnoLiPA was confirmed by sequencing of the 5'NCR, core and NS4 regions. In the haemophiliac samples, sequence analysis of the NS4 region failed to provide evidence that the serotyping assay had incorrectly identified the specificity of the antibody response because of antigenic variation of regions 1 and 2. Amino acid substitutions present in these regions often involved residues of a similar size and charge as those present in the peptides used in the serotyping assay, and therefore unlikely to cause cross reactivity with peptides specific to a different genotype. For example the substitution of valine for alanine at position 1723 in region 2, which was observed in 3/9 type 3 sequences in this study, and the substitution of lysine with arginine in region 1 of GD 4.2. Only one sample in this risk group (GD11) showed an obviously nonconservative amino acid

substitution that might alter the antigenicity of NS4, occurring at position 1691 of region 1 with the substitution of arginine for glutamine.

One possible explanation for the discrepant results between the two assays would be that the ELISA had detected circulating antibody from a previous infection with a different genotype, but despite the high risk of multiple exposure in these individuals, no evidence for infection with more than one genotype was found by PCR with type-specific primers. However, taking into account the high frequency of multiple infections in haemophiliacs (Jarvis *et al.,* 1994), this remains the most likely explanation for the discrepant results in this patient group. Previous studies have demonstrated that the serotyping assay is efficient in the detection of more than one type-specific antibody in serum (Jarvis *et al.,* 1994; Simmonds *et al.,* 1993), although only one serotype was identified in all the haemophiliac samples except for one sample. This suggests that reinfection or the reactivation of genotypes from a multiple infection in these individuals has been recent. Considering that all factor VIII and IX concentrates have been subjected to virus inactivation procedures since 1985, the recent reinfection of these individuals is unlikely. On the other hand, it has been well documented that the major circulating genotype within a multiple infection can often change over a number of years (Jarvis *et al.,* 1994). The genotype identified by PCR based methods generally precedes the corresponding change in type-specific antibody, and has been shown to cause discrepant results between the two methods in previous studies (Jarvis *et al.,* 1994). In addition, the RNA amplified by PCR will represent only the major

circulating virus type in plasma, with the possibility of minor populations in the liver remaining undetected by this method. The absence of a significant level of amino acid variation in NS4 and a risk factor of IVDA in two of the patients with chronic hepatitis C (FD3 and FD4) also suggests that an infection with more than one genotype may explain the discrepant results obtained with these samples.

Antigenic variation

Another possible explanation for misidentification of type-specific antibody by the serotyping assay would be in the event of significant amino acid variation within NS4 antigenic regions 1 and 2 leading to cross reactivity with heterologous type peptides in the assay. Amino acid substitutions were much more frequent in samples from patients with chronic hepatitis C than amongst those from haemophiliacs, and this may have lead to cross reactivity in two of the samples.

Sample FD1 was originally identified as type 1 by InnoLiPA (and confirmed by sequencing), yet showed the presence of type 4 specific antibody in the serotyping assay. Amino acid substitutions were present in both antigenic regions 1 and 2, and the substitution of glutamine for histidine at position 1720 (region 2) also occurs in the corresponding peptide for type 4.

Sample FD2 was identified as type 4 or 5 by InnoLiPA (type 5 by sequencing) but type 1 by the serotyping assay. In region 1, the amino acid sequence corresponded closely to one of the type 1 peptides used in the assay. Only one amino acid substitution was present in this sample in antigenic region 2, with aspartic acid being replaced with glycine at position 1718. Although this change

does not show any similarity to the other peptides used in the assay, it is significant both in size and charge, and may have altered the structure of the antibody sufficiently to prevent the adsorption to the peptides in the assay.

The results in this study suggest that the reliability of serological typing methods may be influenced by the patient groups under investigation, and that PCR based typing methods may be more appropriate in groups of individuals which have been multiply exposed to HCV such as haemophiliacs.

CHAPTER 7

7 DEVELOPMENT OF HCV SEROTYPING ASSAYS BASED ON OTHER REGIONS OF THE GENOME

7.1 INTRODUCTION

The discovery of type-specific epitopes in NS4 has lead to the development of a serotyping ELISA for HCV genotypes 1-3 (Simmonds *et al.,* 1993d) which was subsequently extended to include all six major genotypes (Bhattacherjee *et al.,* 1995). The degree of amino acid variation in NS4 between genotypes resulted in a high level of specificity, with the serotype of antibody detected concordant with genotyping methods in up to 97% samples tested (Chapter 3, Table 3.2.4). However, the sensitivity of this assay was lower, with the detection of type-specific NS4 antibody in 87% samples from HCV infected individuals. The absence of reactivity towards any of the NS4 antigens may be explained by either an immunocompromised state, a recently infected individual prior to seroconversion or the lack of an immune response towards this epitope.

A strong immune response to other regions of the genome has been described as a result of the addition of antigens from core (c22-3), NS3 (c33c) and NS5 to NS4 antigens (c100-3) to second and third generation screening assays. It was discovered that as many as one third of post-transfusion patients with NANB hepatitis did not develop anti-c100 antibody for up to 1 year after infection (Alter *et al.,* 1989), and using a second generation RIBA, it was reported that all highly viraemic patients had a response to core and NS3 antigens, in comparison to 78%

and 85% patients for NS4 (c100-3) and NS5 respectively (Yuki *et al.,* 1994). Similarly, the most common antibodies detected in HCV infected individuals are NS3, core and NS5 (Chien *et al.,* 1992). In addition, a much earlier immune response can be detected towards NS3 (a mean of 91 days after infection) and core (133 days), than for NS4 (143 days) (Lelie *et al.,* 1992).

For these reasons the possibility of a serotyping assay based on other immunodominant regions of the genome having a higher sensitivity than that using NS4 was investigated.

A number of different groups have developed serotyping assays for HCV, the majority of which involve antigens from the NS4 region either alone (Tanaka *et al.,* 1994; Simmonds *et al.,* 1993), or in association with those derived from the core and NS5 regions (Zhang *et al.,* 1995; Dixit *et al.,* 1995). Only one method based exclusively on a region other than NS4 has been described (Machida *et al.,* 1992), which uses peptides from core in an ELISA to distinguish between types 1 and 2. However, despite the success of this assay in the identification of these serotypes in Japanese blood donors, the level of amino acid variation in this region between the remaining genotypes is considered too low to be able to extend this assay further.

In addition to core, the NS3 region is reported as being highly immunogenic (Chien *et al.,* 1992; Yuki *et al.,* 1994), and is often associated with an immune response much earlier after infection than NS4 (Puoti *et al.,* 1992; Lelie *et al.,*

1992). Furthermore, phylogenetic analysis of amplified NS3 sequences has demonstrated a similar level of amino acid variation between genotypes as occurs in the other nonstructural regions (Chan *et al.,* 1992). Recent studies into the reactivity of different genotypes (types 3 and 4) with type 1b specific antigens present in third generation screening assays / RIBA have suggested that reactivity towards NS3 contains a detectable type-specific component (Dow *et al.,* 1996b).

Amongst epitopes identified in NS3, most were conformational as opposed to linear. Little reactivity was observed towards overlapping synthetic peptides in this region, despite the obvious immune response towards NS3 antigens present in screening assays (Khudyakov *et al.,* 1995). Further evidence was obtained when a conformational epitope from amino acids 1363-1454 was recognised by monoclonal antibodies (Mondelli *et al.,* 1994) and by antibody reactivity towards recombinant protein (Claeys *et al.,* 1995). Immunoblot analysis has also revealed at least four linear epitopes in NS3, of which the most immunodominant exists around amino acid residues 1250/1251, and three others within the middle portion of this gene (between amino acids 1250-1334, and 1407-1412) (Hwang *et al.,* 1996).

The combination of an early and frequent immune response to this region, and the apparent type-specific reactivity, has lead to the investigation of NS3 as a possible antigen in the development of a serotyping assay.

7.2 METHODS

Samples

Serum samples were obtained from individuals in which the presence of RNA corresponding to HCV genotypes 1b, 2b and 3a had been previously identified by RFLP analysis of the 5'NCR. Samples NH15 (type 1b), LJ823.2 (type 2b) and LJ516A (type 3a) used for the preparation of NS3 antigens, were obtained from U.K. haemophiliacs (provided by Dr. L. Jarvis). All samples used in ELISA experiments had also been analysed previously using the NS4 1-6 serotyping assay. Samples from individuals infected with type 1 (n=7) were selected from blood donors in Hong Kong, type 2 samples were obtained from either Hong Kong (n=6) or patients with chronic hepatitis C in Pakistan (n=1) and those from individuals infected with type 3 were all obtained from Pakistan (n=5).

Preparation of antigen

Amplification of a 286bp fragment of the NS3 region by PCR was performed using nested primers (Table 2.3; numbers 753, 751 and LP8-10) and the products cloned into pTAg. Sequence analysis of clones was carried out using plasmid primers 8819 and 9130 (Table 2.3). A detailed description of these methods is provided in chapter 2. NS3 recombinant proteins were expressed in *E. coli* and purified at Murex Biotech Ltd, Dartford, U.K.

Microtitre plates were coated with individual type-specific NS3 antigens at Murex Biotech Ltd, at a concentration of 25ng/well (types 1b and 2b) or 200ng/well (type 3a). Serotyping plates were coated with a combination of all three

antigens, each at a concentration of 25ng/well. As the recombinant proteins were expressed in *E. coli*, the addition of bacterial lysate blocker to sample diluent (1:50 dilution) was necessary to prevent the reactivity of antibody produced against this organism with any contaminating *E. coli* proteins remaining after the purification of the antigen.

7.3 RESULTS

7.3.1 DEVELOPMENT OF A SEROTYPING ELISA FOR HCV USING RECOMBINANT PROTEINS FROM THE NS3 REGION

Amplification of NS3

Serum samples from individuals infected with genotypes 1b, 2b and 3a were amplified by PCR in the NS3 region between nucleotides 4417-4703, and cloned into pTAg. Nucleotide sequences of each clone were translated into their corresponding amino acids (residues 1360-1454), and compared to published examples of the same genotype (Figure 7.3.1). The sequences obtained from samples NH15 (type 1b), LJ823.2 (type 2b) and LJ516A (type 3a) were considered to be representative of each genotype, and were therefore selected for expression into recombinant proteins for use as type-specific antigens in an ELISA.

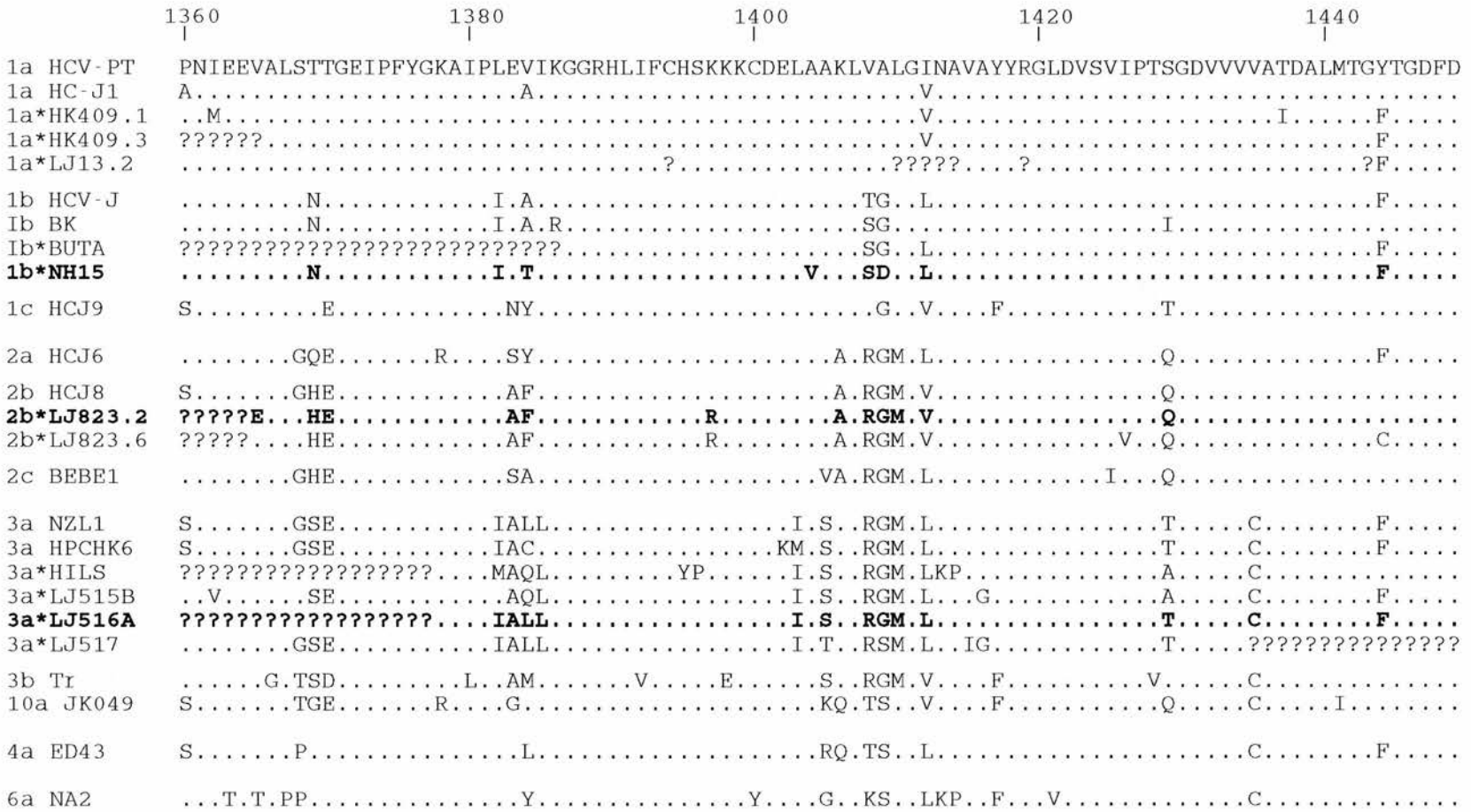
Reactivity of type-specific antibody with NS3 antigens

Figure 7.3.1a COMPARISON OF NS3 AMINO ACID SEQUENCES USED FOR SEROTYPING WITH SEQUENCES SPECIFIC FOR EACH GENOTYPE

A comparison of NS3 amino acid sequences amplified from HCV infected samples in this work (*) with published examples from corresponding genotypes.

Numbers of amino acid residues are shown above HCV-PT

Figure 7.3.1a COMPARISON OF NS3 AMINO ACID SEQUENCES USED FOR SEROTYPING WITH SEQUENCES SPECIFIC FOR EACH GENOTYPE



In order to confirm that each of the recombinant antigens could recognise HCV antibodies, serum samples from individuals infected with genotypes 1, 2 and 3 were incubated on microtitre plates which had been coated with the individual NS3 antigens.

Homologous reactivity (for example, sera from individuals infected with type 1 analysed using a plate coated with type 1 specific antigen) towards each of the three antigens was high, with the majority of absorbance values greater than 2.0 (Table 7.3.1a).

Previously, a 1/40 or 1/20 dilution of serum was necessary to be able to detect a strong signal against NS4 peptides. However, used against NS3 antigen, this dilution consistently produced absorbance values which were too high for our spectrophotometer to read (>2.0). As the design of the serotyping assay is such that it relies on the effective blocking of antibody in solution with competing antigen, both the concentration of antibody (in serum) and competing antigen in each well is critical. To be able to determine the optimum dilution of serum in this assay, samples were tested at dilutions of 1/40, and 1/100 on plates coated with homologous type antigen. In the same experiment, titrations of homologous type competing antigen were also included to determine the level of competition needed for the effective blocking of antibody. The amounts of competing antigens used were the same concentration as that coating each well (1:1), at a ten fold excess (10x) and at a one hundred fold excess to the concentration of protein coating each well (100x).

Table 7.3.1a A COMPARISON OF NS3 TYPE-SPECIFIC ANTIBODY REACTIVITY WITH HOMOLOGOUS TYPE NS3 ANTIGENS

		Homologous type antigen	
		SD*	
Sample	Type	1/40	1/100
HK430	1b	>2.0†	>2.0
HK437	1b	0.218	0.225
HK438	1b	>2.0	>2.0
HK469	1b	>2.0	>2.0
HK347	2a	1.545	0.619
HK8	2b	>2.0	>2.0
HK156	2b	>2.0	>2.0
HK559	2b	>2.0	>2.0
PK24	2	>2.0	>2.0
PK6	3a	>2.0	>2.0
PK9	3a	>2.0	>2.0
PK13	3a	>2.0	>2.0
PK21	3a	>2.0	>2.0
PK36	3a	>2.0	>2.0
PK38	3a	>2.0	>2.0
PK39	3a	>2.0	1.828
PK45	3a	>2.0	>2.0

* Serum Dilution

† Absorbance values correspond to the level of reactivity of samples containing type-specific antibody, with antigens specific to a homologous genotype.

For each genotype, the majority of reactive samples had absorbance values of >2.0 at both the 1/40 and 1/100 dilutions (Table 7.3.1b). It is also noteworthy that certain samples which were tested at a dilution of 1/200 also produced high absorbance values, although the level of antibody in most of these cases was visibly diminished.

In the presence of homologous competition at 1:1 concentration, absorbance values were unchanged (Table 7.3.1b). In the presence of competing protein at a 10x excess, the absorbance values were reduced in some of the samples, indicating that competing protein was effectively blocking antibody reactivity towards antigens coating the plate. However, sufficient levels of antibody remained to produce a clear signal, with absorbance values reduced only to around 1.0, and in many samples (particularly those from individuals infected with type 2), there was no obvious reduction. When incubated with a solution of 100x excess competing antigen, the antibody reactivity was significantly lower in all but one sample, and in the majority of cases, absorbance values were reduced below a level of 0.6, a difference which would be easily distinguishable by eye.

For these reasons, the optimum concentration of serum to be used in this ELISA was 1/100, i.e. 1μl in 100μl sample diluent, with the efficient blocking of antibody achieved in the presence of a 100x excess concentration of competing antigen.

Under these conditions, samples in which very little or no reduction in reactivity was observed were infrequent, and contain an extremely high level of

Table 7.3.1b A COMPARISON OF NS3 ANTIBODY REACTIVITY WITH HOMOLOGOUS TYPE ANTIGEN IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF COMPETING ANTIGEN

Sample	Type	Serum Dilution	Concentration of Competing Antigen* None	1:1	10:1	100:1
HK430	1b	1/40	>2.0	>2.0	>2.0	0.962
		1/100	>2.0	>2.0	1.828	0.729
HK434	1b	1/40	0.746	ND†	0.174	0.060
		1/100	0.280	ND	0.098	0.026
HK461	1b	1/40	1.112	ND	0.334	0.065
		1/100	0.414	ND	0.242	0.028
HK186	2a	1/40	>2.0	>2.0	>2.0	1.681
		1/100	>2.0	>2.0	>2.0	0.701
HK8	2b	1/40	>2.0	>2.0	>2.0	>2.0
		1/100	>2.0	>2.0	>2.0	0.969
HK559	2b	1/40	>2.0	>2.0	>2.0	0.599
		1/100	>2.0	>2.0	0.988	0.276
PK6	3a	1/40	>2.0	>2.0	>2.0	0.771
		1/100	>2.0	>2.0	>2.0	0.381
PK13	3a	1/40	>2.0	>2.0	1.310	0.178
		1/100	>2.0	>2.0	0.816	0.234
PK21	3a	1/40	>2.0	>2.0	>2.0	0.590
		1/100	>2.0	>2.0	>2.0	0.574

* Competing antigens were of a homologous genotype to that identified in the sample under investigation, and are represented as an excess level to that coating the wells of the microtitre plate. For example, 10:1 concentration represents the addition of a ten fold excess level of protein to that present coating each well.

† Sample not done

NS3 antibody. Although in these cases a 1:1000 concentration of competing antigen would most likely be effective, this level of competition would be impractical in a final version of the assay due to the volumes of competition needed in each well affecting serum concentrations. In practice, these samples would be repeated at a higher dilution.

NS3 Serotyping ELISA

Using the results obtained so far, a competitive assay using NS3 antigens for the detection of type-specific antibody was designed. Microtitre plates were coated with equal concentrations of NS3 proteins corresponding to genotypes 1, 2 and 3, and serum samples incubated at a 1/100 dilution. Solutions containing heterologous type competing antigens at a 100 fold excess concentration were added to the typing wells, and a solution containing all antigens to the fully blocked negative control well, as previously described for the NS4 serotyping ELISA (section 2.1.2).

From seven type 1 samples analysed, only four were typed correctly by the assay, of which two displayed high levels of background reactivity (Table 7.3.1c), making identification difficult by eye. Two samples (HK434 and HK461) were reactive in the unblocked well, but showed no reactivity in the typing wells, and one sample was unreactive towards the NS3 antigens.

In all but one of the samples from individuals infected with type 2, antibody of the corresponding serotype was detected by the assay. However, in 3/6 of these

Table 7.3.1c ANALYSIS OF SERUM SAMPLES FROM INDIVIDUALS INFECTED WITH TYPES 1-3 USING THE NS3 SEROTYPING ASSAY

		Typing Well					
Sample	Type	UB	FB	1	2	3	Interpretation
HK434	1b	0.753	0.141	0.135	0.208	0.201	NTS
HK437	1b	0.045	0.012	0.047	0.107	0.067	NR
HK438	1b	>2.0	0.423	1.718	0.401	0.700	1
HK461	1b	0.404	0.034	0.058	0.068	0.062	NTS
HK467	1b	>2.0	1.040	>2.0	1.858	1.104	1*
HK363	1a	0.941	0.118	0.774	0.112	0.124	1
HK406	1a	>2.0	0.637	1.338	0.738	0.652	1*
HK186	2a	>2.0	0.329	0.111	0.402	0.487	2+3
HK315	2a	>2.0	0.089	0.074	0.162	1.029	3
HK409	2a	>2.0	0.839	0.842	>2.0	1.210	2*
HK8	2b	>2.0	0.309	0.368	0.646	0.796	2+3
HK156	2b	>2.0	1.343	1.441	>2.0	1.769	2*
HK559	2b	1.258	0.068	0.093	0.780	0.162	2
PK24	2	>2.0	0.312	0.252	1.180	>2.0	2+3
PK9	3a	>2.0	0.546	0.313	0.431	1.829	3
PK13	3a	0.284	0.049	0.034	0.059	0.056	NTS
PK21	3a	1.730	0.314	0.254	0.410	0.694	3*
PK36	3a	>2.0	0.178	0.044	0.126	1.056	3
PK38	3a	0.104	0.070	0.048	0.052	0.058	NTS

UB; Unblocked control well
FB; Fully Blocked control well

NTS; Non type-specific reactivity
NR; Non reactive

* High levels of background reactivity

samples a positive reaction was also evident in the third typing well, suggesting that both type 2 and 3 specific antibody was present. The NS3 serotyping ELISA was able to detect type-specific antibody in 3/5 samples from individuals infected with type 3, with the remaining two sera showing non-type-specific reactivity. These results suggest that a certain level of cross reactivity may exist between types 2 and 3, and although the overall detection of NS3 antibody by the serotyping ELISA was high, its ability to distinguish between different serotypes was somewhat less successful.

Investigation of cross reactivity between genotypes 2 and 3

In order to investigate the possibility of cross reactivity between types 2 and 3 further, the ability of type 3 competing antigen to block the reactivity of type 2 antibody with type 2 antigen on the solid phase was examined, and vice versa. In total, 15 samples from individuals infected with either type 2 or 3 were reacted against homologous type antigen, in the presence of (i) no competing antigens, (ii) type 2 competition only, (iii) type 3 competition only and (iv) a competing solution containing both type 2 and 3 protein (Table 7.3.1d). In the absence of cross reactivity, a positive result would be expected to be confined to the unblocked well and the well containing heterologous type competing antigen, with lower absorbance values present in the wells containing competition with type homologous antigen.

Table 7.3.1d A COMPARISON OF THE LEVELS OF CROSS REACTIVITY BETWEEN TYPES 2 AND 3 WITH NS3 AND NS3/NS4 ANTIGENS

		NS3 ELISA					NS3/NS4 ELISA				
		Competing antigen					Competing antigen				
Sample	Type	UB	2	3	2+3	Result	UB	2	3	2+3	Result
HK186	2a	>2.0	0.777	0.961	0.803	NTS	1.783	0.444	0.873	0.229	2
HK315	2a	>2.0	1.249	0.336	0.406	3	0.298	0.027	0.030	0.008	NTS
HK409	2a	>2.0	1.473	>2.0	1.801	NTS	0.806	0.037	0.125	0.038	2
HK8	2b	>2.0	0.848	0.757	0.710	NTS	1.226	0.043	0.041	0.027	NTS
HK156	2b	>2.0	1.424	>2.0	1.982	2/NTS	1.558	0.231	0.836	0.179	2
HK559	2b	>2.0	0.358	1.093	0.509	2	0.622	0.021	0.179	0.025	2
PK24	2	>2.0	1.738	1.460	1.113	NTS	1.455	0.157	1.348	0.266	2
PK6	3a		Not Done				1.540	1.112	0.144	0.080	3
PK9	3a	>2.0	1.489	0.121	0.147	3	1.171	1.028	0.182	0.106	3
PK13	3a	0.354	0.010	0.001	0.006	NTS	0.786	0.963	0.028	0.040	3
PK21	3a	1.353	0.197	0.038	0.060	NTS	0.497	0.071	0.040	0.024	NTS
PK36	3a	>2.0	>2.0	0.055	0.135	3	1.043	0.595	0.030	0.030	3
PK38	3a	0.006	0.014	0.001	0.001	NR	0.252	0.326	0.037	0.041	3
PK39	3a	0.790	0.385	0.006	0.002	3	0.278	0.143	0.016	0.033	3

UB Unblocked positive control well
NTS Non type-specific reactivity (appendix 1.0)
NR Non reactive

All type 2 sera were positive in the unblocked well with the majority of absorbance values >2.0, confirming the presence of a high titre of NS3 antibody in these samples. Although a higher level of reactivity was observed in the well containing only type 3 competing antigen in 5/15 samples, the remaining samples analysed presented a reduction in reactivity with all three combinations of competing antigen. These results (Table 7.3.1d) confirm that both type 2 and 3 protein is able to recognise NS3 antibody produced against certain type 2 isolates.

A positive reaction in the unblocked well confirmed the presence of NS3 antibody in 13/15 samples from individuals infected with type 3. Type 3 specific antibody was correctly identified by a reaction in the well containing type 2 competition alone in 8/13 samples analysed, with the complete eradication of reactivity in any of the typing wells observed in the remaining 5 sera. Interestingly, the "unblocked" absorbance values for samples in which the competing proteins removed all reactivity were lower than with the other samples. Although cross reactivity also occurs between type 2 protein and type 3 antibody, this seems to be less frequent than that observed between type 3 protein and type 2 antibody.

The development of a serotyping ELISA using NS3 recombinant protein as antigen was successful in increasing the sensitivity towards type-specific antibody, but lacked the specificity of NS4 in the ability to distinguish between types 2 and 3. In an effort to combine the sensitivity of the NS3 assay with the specificity of the NS4 assay, I reasoned that both antigens together could compensate for the

cross reactivity observed using NS3 alone, and therefore result in an improved version of the serotyping ELISA. This was investigated by the production of type-specific fusion proteins consisting of both the NS3 and NS4 antigenic regions in the form of a single recombinant protein, for use in a competitive serotyping ELISA.

7.3.2 THE DEVELOPMENT OF A SEROTYPING ASSAY USING A COMBINATION OF NS3 AND NS4 ANTIGENS

(1) PREPARATION OF NS3/NS4 ANTIGENS

Cloning of NS3 and NS4 regions

Samples in which the presence of genotypes 1b, 2b and 3a had been confirmed by RFLP were amplified by PCR in the NS3 and NS4 regions using type-specific primers (Table 2.3; LP2-7 and LP11-16) containing complementary restriction sites at each end (Figure 7.3.2a). Each PCR product was cloned into pTAg, and analysed by sequencing to confirm the genotype. The individual clones (NS3 = 286bp; NS4 = 98bp) were cleaved from the plasmid DNA using the relevant restriction enzymes and ligated together prior to a further ligation reaction to insert the 384bp fragment into a pTAg backbone with prepared corresponding restriction sites (section 2.6).

Each NS3/NS4 clone (renamed FP1-3) was analysed by sequencing, and translated into the corresponding amino acids to confirm that both regions were in frame prior to expression (Figure 7.3.2a). FP1, 2 and 3 were constructed from isolates NH15 (type 1b), LJ823 (type 2b) and LJ516 (type 3a).

Protein expression

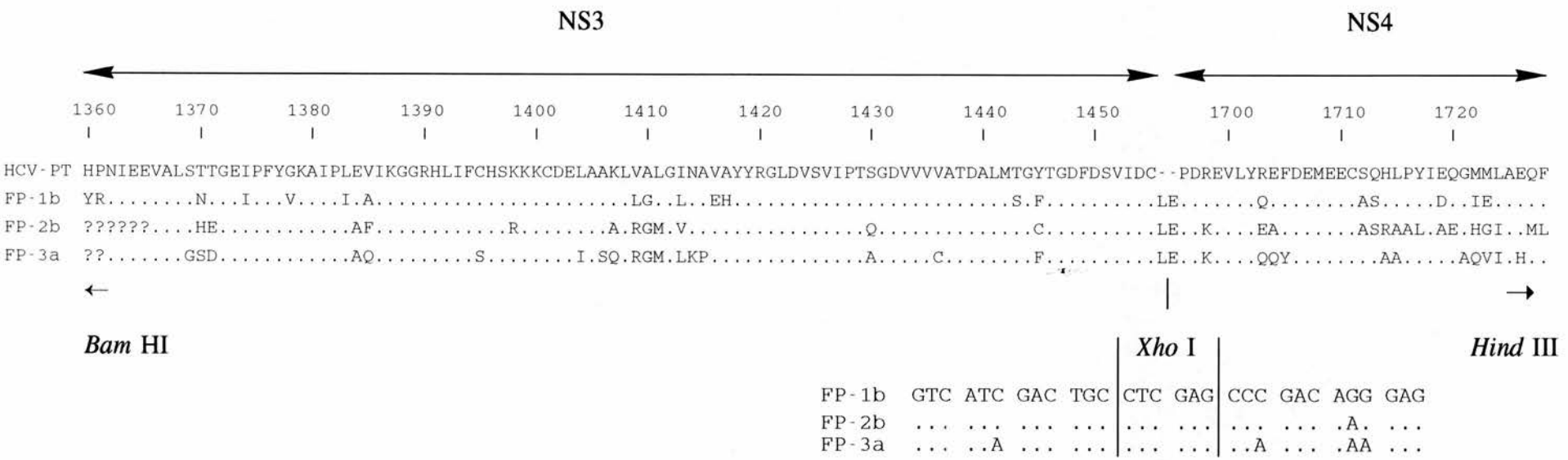
The NS3/NS4 clones were subcloned into pRSET for expression in *E. coli* (section 2.6). Expressed proteins (128 amino acids in length) were visualised by

PAGE (Figure 7.3.2b) in the supernatant from the bacterial culture, and their identity confirmed by Western blotting (section 2.6.5) (Figure 7.3.2c).

Each protein was purified using metal chelate affinity chromatography (section 2.6). Proteins were recovered with ten consecutive elutions with 300mM imidazole, and identified in the third elution (E3) by PAGE (Figure 7.3.2d).

The concentration of each protein in E3 was calculated by PAGE, with a comparison of the band density produced from 1μl (or 0.1μl) E3 with standard concentrations of BSA. The concentration of type 1 protein was 15 mg/ml, type 2 was the highest concentration at 65 mg/ml and a relatively low yield of type 3 protein was obtained at 3 mg/ml.

Figure 7.3.2a AMINO ACID SEQUENCES OF NS3/NS4 CLONES USED FOR THE PREPARATION OF ANTIGENS FOR HCV SEROTYPING

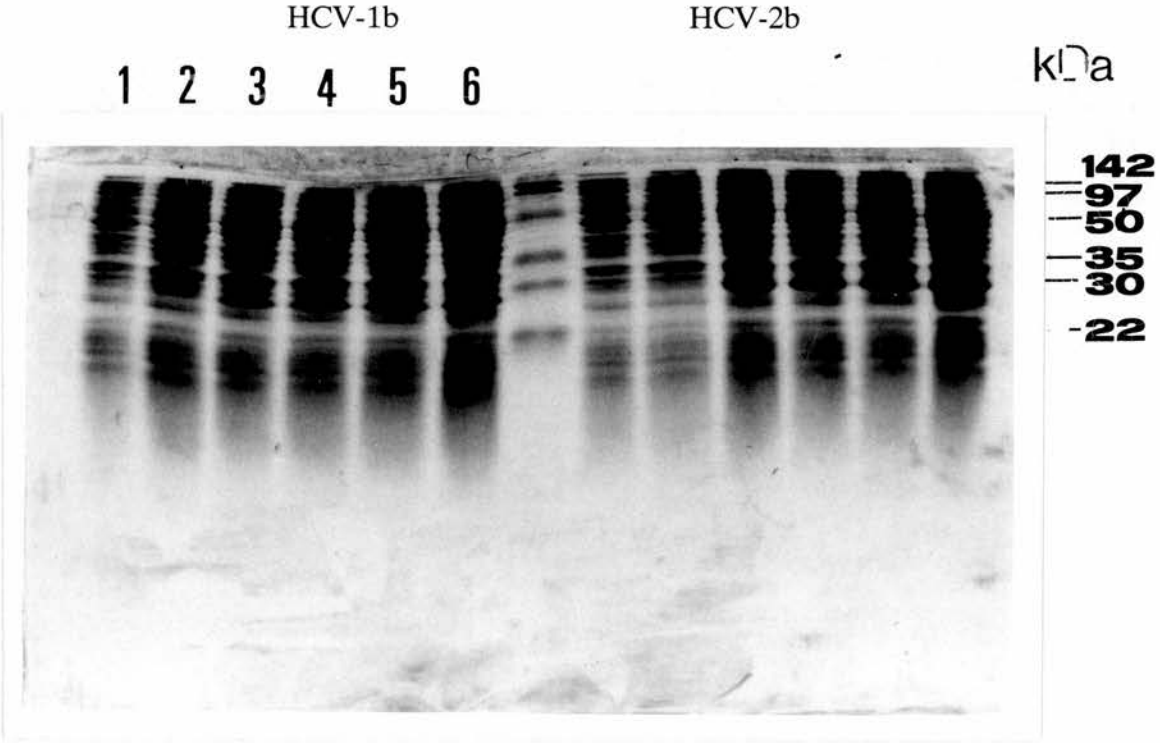


Amino acid positions are numbered above the prototype sequence (HCV-PT)

"?" Sequence not determined

"." Sequence identity to HCV-PT

Figure 7.3.2b IDENTIFICATION OF EXPRESSED HCV NS3/NS4 PROTEIN IN *E. COLI* CULTURE SUPERNATANT

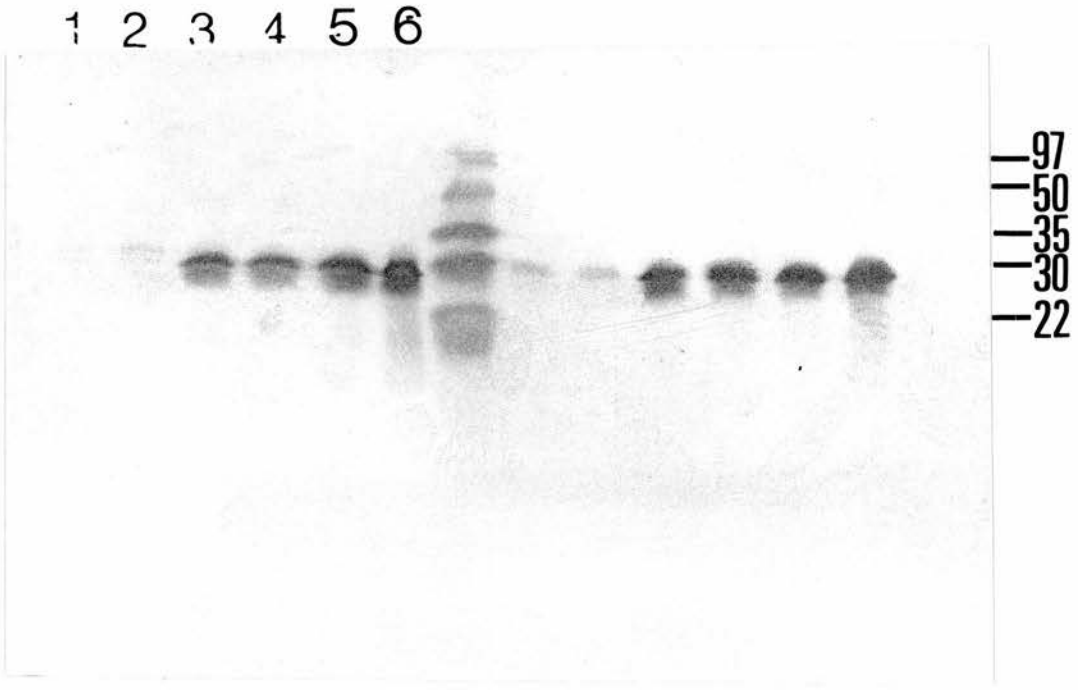


Pilot expression of HCV type 1b and type 2b NS3/NS4 fusion proteins in *E. coli.*

Lanes 1-6 represent samples taken from the *E. coli* culture supernatant at the following time points;

Lane 1= addition of IPTG (0 hours)
Lane 2= Induction with M13/T7 bacteriophage (1 hour)
Lane 3= 2 hours post induction
Lane 4= 3 hours post induction
Lane 5= 4 hours post induction
Lane 6= 16 hours (overnight) post induction

Figure 7.3.2c IDENTIFICATION OF HCV NS3/NS4 PROTEINS BY WESTERN BLOTTING



Identification of expressed NS3/NS4 fusion proteins for type 1b and type 2b by Western blotting.

Lanes 1-6 correspond to the time point for which the samples were taken from the *E. coli* culture during a pilot expression. Lane 1= addition of IPTG (0 hours); Lane 2= induction with M13/T7 bacteriophage (1 hour); Lane 3= 2 hours post induction; Lane 4= 3 hours post induction; Lane 5= 4 hours post induction; Lane 6= 16 hours post induction

Figure 7.3.2d

Identification of HCV NS3/NS4 fusion proteins for type 1b (A) and type 2b (B) by metal chelate affinity chromatography.

Lanes 1-6 correspond to samples taken from the flowthrough after washing the following the columns with the following solutions;

Lane 1= *E. coli* culture supernatant flowthrough
Lane 2= Binding buffer wash #1
Lane 3= Binding buffer wash #2
Lane 4= Binding buffer wash #3
Lane 5= 10mM imidazole wash
Lane 6= 30mM imidazole wash

Lanes E1-E7 correspond to samples taken from the flowthrough from consecutive elution washes with 300mM imidazole.

Figure 7.3.2d IDENTIFICATION OF HCV NS3/NS4 PROTEINS DURING PURIFICATION BY METAL CHELATE AFFINITY CHROMATOGRAPHY

(2) ANTIBODY REACTIVITY TOWARDS NS3/NS4 ANTIGENS

To enable a direct comparison of the performance of this assay with that using NS3 antigen alone, the same samples were used for each analysis. Similarly, microtitre plates were coated with the same concentration of each antigen, and competing antigen solutions contained a 100x excess amount of antigen. All other conditions, for example reagents and incubation times also remained as standard.

Homologous and Heterologous reactivity

The reactivity of antibody with antigen corresponding to the same genotype was high for each of the samples from individuals infected with types 1, 2 and 3. In the absence of competing antigen, the reactivity of type-specific antibody with antigens specific to heterologous genotypes was also observed, although the absorbance values in such cases were significantly lower. The lower level of heterologous reactivity was also observed in type 2 and 3 samples which had been cross reactivity using NS3 antigens alone. Using NS3/NS4 antigens, lower absorbance values were produced during reactions between type 3 antibody and type 2 antigen, and vice versa (Table 7.3.2a).

Serum Titrations

Samples were analysed at dilutions of 1/40, 1/100 and 1/200. Identical samples produced slightly lower absorbance values with NS3/NS4 antigens than previously detected with NS3 only, but this may have been caused by the longer

Table 7.3.2a A COMPARISON OF ANTIBODY REACTIVITY WITH HOMOLOGOUS AND HETEROLOGOUS TYPE NS3/NS4 ANTIGENS

		Type 1 antigen		Type 2 antigen		Type 3 antigen	
		SD*		SD		SD	
Sample	Type	1/40	1/100	1/40	1/100	1/40	1/100
HK434	1b	0.380	0.137	0.550	0.280	0.276	0.151
HK438	1b	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
HK461	1b	1.203	0.539	0.266	0.126	0.479	0.197
HK469	1b	1.576	1.199	>2.0	1.756	1.592	1.087
HK186	2a	0.785	0.682	>2.0	>2.0	>2.0	>2.0
HK315	2a	0.158	0.145	1.936	1.293	1.534	1.275
HK8	2b	1.390	1.284	>2.0	>2.0	>2.0	1.837
HK156	2b	0.283	0.108	>2.0	>2.0	1.799	1.587
PK6	3a	0.130	0.088	>2.0	>2.0	>2.0	>2.0
PK9	3a	0.145	0.070	1.667	1.270	>2.0	>2.0
PK13	3a	0.053	0.023	0.507	0.229	>2.0	1.740
PK21	3a	0.380	0.191	1.687	0.941	1.559	1.043

* Serum Dilution

duration of storage in combination with additional freeze/thaw cycles leading to a reduction in antibody concentration. The optimum dilution of serum for reactivity was 1/100, with absorbance values generally between 1.0 and 2.0 (Table 7.3.2a). In addition, the reactivity of the majority of samples at this dilution was reduced to background levels in the presence of 100x excess competing antigen.

Investigation of cross reactivity between genotypes 2 and 3

The frequency of cross reactivity between type 2 or type 3 specific antibody with heterologous competing antigen was investigated using identical samples and experimental conditions as for NS3 (section 7.3.1). A comparison of the results from seven samples from individuals infected with each genotype are compared in Table 7.3.1d.

Using NS3/NS4 antigen, cross reactivity between type 2 antibody and type 3 competing antigen was less frequent than with NS3 alone. Only 1/7 sera (HK8) tested using the NS3/NS4 antigens produced misleading results due to cross reactivity with heterologous competing antigen, in comparison with 4/7 identical samples with NS3. The use of a combination of both antigens has compensated for the cross reactivity observed with NS3 alone in two samples, HK186 and PK 24 (Table 7.3.1d). The results of sample HK315 are regarded as inconclusive, as analysis with the NS4 serotyping assay was unable to confirm the presence of type 2 specific antibody, despite the identification of this genotype by RFLP.

An even greater improvement in the level of type specific reactivity was observed with samples from individuals infected with type 3. All samples but one were reactive in the well containing only homologous competing antigen, whereas only three samples failed to cross react with type 2 competition using NS3 alone. In particular, sample PK38 had previously shown no reactivity towards NS3 antigens, suggesting that the reactivity observed against NS3/NS4 antigens was directed towards the NS4 epitope of the fusion protein. Similarly, both an increase in reactivity and specificity was observed for sample PK13 using NS3/NS4 antigens.

NS3/NS4 Serotyping ELISA

Samples from individuals infected with each of genotypes 1, 2 or 3 were analysed using a serotyping ELISA based on the NS3/NS4 antigens, with a higher number of samples producing accurate results than in a previous study of the same samples using the serotyping assay based on NS3 antigens (Table 7.3.2b).

Type-specific antibody was detected in 5/6 samples from individuals infected with type 1, of which two (HK437 and HK461) had previously been untypeable using the NS3 assay (Table 7.3.2c). Despite the use of type 1b antigen, two sera from type 1a infected individuals were also serotyped successfully. Similarly, much clearer results were obtained with samples containing type 2 antibody, with 5/6 samples identified correctly by the assay. Unlike the results obtained with identical samples using the NS3 assay, background absorbance levels

were relatively low and the results clearly distinguishable by eye. In addition, two samples producing non type-specific reactivity with NS3 were identified correctly using the NS3/NS4 ELISA (Table 7.3.2c). The highest level of specificity by this assay was observed with samples from individuals infected with type 3. From a total of seven samples analysed, type-specific antibody was detected in all but one (Table 7.3.2b). This sample also had a higher level of reactivity in the typing well corresponding to type 3 than in the other two wells, although the difference in absorbance was too low to clearly define this serotype.

Table 7.3.2b ANALYSIS OF SERUM SAMPLES FROM INDIVIDUALS INFECTED WITH TYPES 1-3 USING THE NS3/NS4 SEROTYPING ASSAY

		Typing Well					
Sample	Type	UB	FB	1	2	3	Interpretation
HK434	1b	0.270	0.000	0.054	0.061	0.016	NTS
HK437	1b	1.754	0.317	1.725	0.483	0.383	1
HK461	1b	0.330	0.141	0.373	0.203	0.231	1*
HK363	1a	1.012	0.206	0.646	0.290	0.309	1
HK406	1a	>2.0	0.128	0.493	0.199	0.362	1*
HK469	1b	>2.0	0.225	0.723	0.508	0.566	1*
HK186	2a	>2.0	0.301	0.220	1.574	0.240	2
HK409	2a	1.843	0.040	0.047	0.213	0.027	2
HK8	2b	1.971	0.067	0.039	0.047	0.084	NTS
HK156	2b	>2.0	0.597	0.438	1.488	0.594	2
HK559	2b	1.158	0.027	0.025	0.215	0.025	2
PK24	2	>2.0	0.650	0.680	1.940	1.680	2+3
PK6	3a	1.955	0.095	0.104	0.102	1.350	3
PK9	3a	>2.0	0.388	0.390	0.373	1.744	3
PK13	3a	1.315	0.071	0.062	0.069	0.965	3
PK21	3a	1.426	0.136	0.165	0.267	0.316	NTS
PK36	3a	1.971	0.086	0.120	0.095	1.541	3
PK38	3a	0.499	0.034	0.040	0.039	0.380	3
PK39	3a	0.530	0.029	0.018	0.006	0.390	3

UB Unblocked positive control well
FB Fully blocked negative control well

NTS Non type-specific reactivity

* High levels of background reactivity

Table 7.3.2c A COMPARISON OF THE ABSORBANCE VALUES OF SAMPLES PRODUCING DIFFERENT RESULTS IN THE NS3 AND NS3/NS4 SEROTYPING ASSAYS

		NS3 SEROTYPING						NS3/NS4 SEROTYPING					
Sample	Type	UB	FB	1	2	3	Result	UB	FB	1	2	3	Result
HK437	1b	0.045	0.012	0.047	0.107	0.067	NR	1.754	0.317	1.725	0.483	0.383	1
HK461	1b	0.404	0.034	0.058	0.068	0.062	NTS	0.330	0.141	0.373	0.203	0.231	1
HK186	2a	>2.0	0.329	0.111	0.402	0.487	NTS	>2.0	0.301	0.220	1.574	0.240	2
HK156	2b	>2.0	1.343	1.441	>2.0	1.769	2*	>2.0	0.597	0.438	1.488	0.594	2
PK13	3a	0.284	0.049	0.034	0.059	0.056	NTS	1.315	0.071	0.062	0.069	0.965	3
PK38	3a	0.104	0.070	0.048	0.052	0.058	NTS	0.499	0.034	0.040	0.039	0.380	3

UB Unblocked positive control well
FB Fully blocked negative control well

NR Non reactive
NTS Non type-specific reactivity (appendix 1.0)
* High levels of background reactivity

7.4 DISCUSSION

The NS4 serotyping assay has been shown to be successful in the detection of HCV type specific antibody. However, the high level of specificity may be compromised by a reduced sensitivity in some individuals who lack an immune response to NS4. The hypothesis that a more sensitive ELISA could be designed using antigens from the NS3 region followed a number of reports suggesting that NS3 was associated with both a more frequent (Chien *et al.,* 1992; Yuki *et al.,* 1994) and earlier (Lelie *et al.,* 1992) immune response in HCV infected individuals. In addition, a significant level of type-specific reactivity has been identified by certain genotypes towards type 1 NS3 antigens in second and third generation screening assays (Dow *et al.,* 1996b).

Preliminary experiments demonstrated that a significantly higher level of reactivity was produced with NS3 antigens in comparison to NS4. Indeed, up to a five fold increase in antibody concentration was observed in many sera, as absorbance values >2.0 were detected from a 1/200 serum dilution against NS3 proteins in comparison to 1/40 dilution against the same concentration of NS4 peptides.

The ability of the NS3 serotyping assay to distinguish between antibody serotypes was less successful. Although a number of the samples analysed were identified correctly, particularly those from individuals infected with type 1, a significant number of samples from individuals infected with types 2 or 3 produced inaccurate results. Subsequent investigations confirmed that cross reactivity between

types 2 and 3 in some infections, but not others, could take place, and that this seemed to occur more frequently with antibody generated during type 2 infections than type 3. A comparison of NS3 amino acid sequences (Figure 7.3.1) revealed that the motif "arginine-glycine-methionine" (amino acids 1408-1410) was present in both these genotypes but different in others. One possibility may be that in samples which are cross reactive, this common motif represents the major epitope towards which the immune response is directed, whereas in cases where no cross reactivity between the two genotypes is observed, other more type specific epitopes are immunodominant, for example that existing at amino acids 1382-1386 (type 2 = "LAFI"; type 3 = "IALL") or at amino acids 1404-1407 (type 2 = "LAAA"; type 3 = "IASK"). The higher frequency of cross reactivity observed in type 2 infections may reflect a more immunodominant role of amino acids 1408-1410 in type 2 isolates.

The relatively high concentrations of NS3 antibody in HCV infected individuals, together with a more frequent immune response in comparison to NS4 made this antigen an ideal candidate for the development of a more sensitive serotyping ELISA. Similarly, the appearance of NS3 antibody significantly earlier during seroconversion would theoretically allow the determination of HCV serotype much earlier during an infection, which may be advantageous in clinical practice as both the genotype of HCV and duration of infection are factors associated with a sustained response to interferon. However, despite these many favourable

characteristics associated with NS3, the significant level of cross reactivity observed between types 2 and 3 indicates that this antigen is unsuitable for the detection of type-specific antibody.

In an effort to combine the sensitivity of NS3 antigens with the specificity of NS4 within a single serotyping ELISA for HCV, the two regions were united in the form of a single recombinant protein. The high sensitivity of NS3 was maintained in this assay, with sufficient concentrations of antibody present in a 1/100 dilution of serum (1μl per well) to ensure clear results by the majority of samples. In a number of samples, the incorporation of NS4 was able to compensate for cross reactivity observed between types 2 and 3 in the NS3 region alone (Table 7.3.2c), resulting in the detection of type-specific antibody in a higher number of samples.

A further advantage of the combined antigen could be in the event of an individual in which an immune response towards one of these regions was absent. This was demonstrated in sample HK437, which was unreactive towards NS3 antigens alone, and yet the presence of type 1 specific antibody was clearly detected using NS3/NS4 antigen. There have been a number of reports of an earlier (Lelie *et al.,* 1992), and more frequent immune response towards NS3 in comparison to NS4 (Chien *et al.,* 1992; Yuki *et al.,* 1994), suggesting that a higher number of samples will rely on the reactivity to the NS3 region alone. In some of these cases, the cross reactivity between types 2 and 3 may still present a problem,

however, the identification of type-specific antibody in a significant number of NS4 "non reactive" samples would become a possibility with this new ELISA.

Clearly a much higher number of samples must be analysed before the exact role of the NS3/NS4 serotyping ELISA can be assessed, including particularly those which had produced non type-specific results, or were unreactive with the NS4 assay. Furthermore, the addition of antigens for other HCV subtypes may increase both the sensitivity and specificity of this assay, and should therefore be investigated.

CHAPTER 8

8 DISCUSSION ; PROBLEMS AND FUTURE DEVELOPMENTS OF THE HCV SEROTYPING ASSAY

Diversity of HCV

The aim of this thesis was to develop a sensitive ELISA which would be able to detect and distinguish between the broad range of HCV genotypes distributed worldwide. Prior to this work, a competitive ELISA based on antigenic regions of the NS4 genome, was only capable of detecting HCV serotypes 1-3, and therefore was only successful in countries such as the U.S.A, Japan and Northern/Western Europe, where these genotypes predominated.

In this study the assay was extended to incorporate antigens for genotypes 4, 5 and 6, resulting in an assay which could be used effectively in the detection of the full range of HCV genotypes occurring worldwide (Chapter 3). There was no decrease in the sensitivity of the assay for types 1-3, while a large proportion of samples from individuals infected with types 4, 5 and 6 were correctly identified. However, studies using samples from HCV-infected individuals in certain countries, such as Pakistan and Egypt, revealed a higher frequency of non-specific reactivity as compared with studies from Western countries (Chapter 3). The most likely explanation for the reduced specificity of the assay in these areas is the occurrence of additional subtypes for which the antigens in the assay are less cross-reactive. In order to be able to increase the sensitivity of the assay towards these subtypes, it would be necessary to incorporate additional subtype-specific

NS4 peptides. For example, the assay already incorporates peptides for subtypes 2a, 2b and 2c, resulting in an increase in the sensitivity and specificity of the assay for this type 2. However, given the vast array of subtypes identified in recent years, it may not be possible to extend this to the full range of subtypes currently identified for this and other genotypes. For example, up to 17 subtypes of type 3 have been identified, and it would be impossible to incorporate this number of antigens (17 x 2 = 34) into the assay because of limitations in the amount of each antigen binding to the plate. In addition, because each antigen is present at 100x the coating concentration in the competition mix, this may exagerate cross-reactivity and lead to a reduction in the specificity of the assay for other genotypes. One alternative would to be to produce a kit which contains antigens specific to the genotypes prevalent in the geographical area under investigation, but the cost of development of a separate assay for each geographical region would be prohibitive.

Serotyping analysis of samples from individuals infected with type 6 group variants was successful in only some of the samples, as a result of amino acid variation present within the NS4 antigenic regions corresponding to the peptides used in the ELISA (Chapter 5). Although it may be possible to increase the sensitivity of the assay towards type 6 group variants by incorporation of the corresponding peptides, the relatively confined geographical distribution of these isolates in South East Asia fails to justify such measures. Similar problems are faced by current genotyping methods. Type 6 group variants are currently unable to be identified by many assays which are based on the 5'NCR such as RFLP

analysis and InnoLiPA, as these isolates show a high degree of sequence similarity to type 1 in this region. Owing to the extreme diversity of HCV, it is unlikely that a genotyping assay which is able to detect all HCV variants will ever be produced.

Clinical Significance of a Serotyping Assay for HCV

The clinical significance of different HCV genotypes is currently an active area of research, and the vast majority of reports suggest that infection with type 1 is associated with a lower frequency of sustained response to Interferon treatment than infection with types 2 or 3. One important application of the serotyping assay in clinical practice is therefore in the selection of HCV infected individuals for Interferon treatment, as this drug is expensive, and often associated with severe side effects. Although Interferon therapy is currently based on PCR analysis, the use of the serotyping assay would provide a relatively inexpensive and rapid method of genotype determination from large patient groups prior to confirmation by PCR.

The clinical significance of genotypes 4-6 is relatively unknown, with only a few papers to date, suggesting that infection with type 4 may be associated with a poor response to interferon (Lau *et al.,* 1996; El Zayadi *et al.,* 1996). The 1-6 serotyping assay may play an important role in such studies, in the determination of HCV genotypes causing infection in patients undergoing treatment. However, in the event that a lower frequency of sustained response is associated with only type 1, the development of an ELISA which is able to distinguish type 1 from any of the other major genotypes may be more relevant in clinical situations.

Some groups have suggested that non-responsiveness to interferon treatment is specifically associated with type 1b. However, many of these studies have been performed in countries where this genotype predominates, such as Japan, and so direct comparison with subtype 1a has not always been possible. If this difference is confirmed, there would be a greater demand for assays which are able to distinguish between type 1 subtypes, in addition to other genotypes. Insufficient amino acid variation is present in NS4 antigenic region 1 and 2 to be able to differentiate between subtypes of type 1, and it would be necessary to investigate the possibility of using additional antigens for this purpose. A comparison of amino acid sequences in NS4 antigenic region 3 revealed some variation between types 1a and 1b, but a low frequency of antibody reactivity was observed in this study when using this antigen in a serotyping ELISA (chapter 4).

In conclusion, the 1-6 serotyping assay may play an important role in the identification of HCV genotypes causing infection in individuals who are under consideration for interferon treatment. In addition, the ability of the assay to identify genotypes 4-6 will be important in future studies concerning the clinical significance of infection with these genotypes.

Improvements to the HCV Serotyping Assay

In an attempt to increase the sensitivity of the serotyping assay, the ability of antigens from the NS3 region for the detection of type-specific antibody was investigated (Chapter 7). A higher reactivity of NS3 antibody was observed in the

majority of samples compared with NS4, with strong reactivity produced from as little as 1μl of serum. This would be particularly useful for the analysis of samples from immunocompromised patients, in which the concentration of antibody is often low, and may improve the sensitivity of the serotyping ELISA when analysing groups of patients such as haemophiliacs or post-transplant patients on immunosuppressant drugs.

Although the sensitivity of the NS3 serotyping assay was greater in comparison with the NS4 assay, the specificity of type-specific NS3 antigens was much lower due to a high frequency of cross-reactivity between types 2 and 3. This problem was addressed by the incorporation of antigens from both regions in the form of a NS3/NS4 fusion protein. In this format, the specificity of the assay was increased, with the successful detection of type-specific antibody in some samples which had produced non-type-specific results using NS3 antigens alone. Although the specificity of the NS3/NS4 serotyping assay for the identification of types 2 and 3 is unlikely to exceed that of the NS4 serotyping assay, the sensitivity towards types 1, 2 and 3 has been increased.

Possible future developments of the serotyping assay

Future development of the NS3/NS4 serotyping assay might include the incorporation of antigens for genotypes 4, 5 and 6, and an investigation into the potential of antigens from individual subtypes. One advantage of the incorporation of the NS3 (c33c) antigen into second generation screening assays has been in the

earlier detection of antibody during HCV infection following seroconversion, as this protein is often the first to generate an immune response (Puoti *et al.,* 1992; Lelie *et al.,* 1992). This may also apply to the NS3/NS4 serotyping assay, and it would be interesting to analyse samples from HCV positive individuals which had previously been unreactive on the NS4 serotyping assay. The number of false negative results on the serotyping assay could theoretically be reduced further by the incorporation of additional antigens, such as core, E1 and NS5. However, the core region is relatively conserved between genotypes, and has only been successful in the detection of types 1 and 2 (Machida *et al.,* 1992).

This work represents a significant improvement of serotyping assays for HCV, and may prove valuable in clinical practice for the selection of patients for drug therapy.

SUMMARY

- This work has resulted in the successful development of a competitive ELISA which is able to distinguish between all six major genotypes of HCV.
- The application of this assay to samples obtained from a number of different geographical areas has provided information about the HCV genotype distribution in various countries, including Hong Kong, Egypt, Norway, Pakistan and Taiwan.
- HCV serotyping has also contributed to both epidemiological and clinical studies, including the identification of a possible link between type 6a infection in Hong Kong and the transmission of this genotype by drug abuse.

- The sensitivity of the existing serotyping assay was improved by the addition of antigen (in the form of recombinant protein) to the NS3 region. This has resulted in an assay which is able to distinguish between HCV serotypes 1-3 from only 1μl serum.

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APPENDIX

1.0 GLOSSARY OF TERMS COMMONLY USED THROUGHOUT THIS WORK.

(a) Sensitivity

The sensitivity of the serotyping assay is a calculation of the percentage of samples analysed in which NS4 antibody can be detected. This value is calculated by dividing the number of samples which are reactive to the NS4 antigens by the total number of samples analysed.

$$\text{i.e.} \quad \frac{\text{No. samples reactive on serotyping assay*}}{\text{Total number of samples analysed}}$$

* This includes samples which were reactive to the NS4 antigens collectively but for which type-specific antibody was not obvious ("NTS" results).

(b) "Specificity" and "Concordance"

The serotyping assay shows a high level of "specificity" (or is highly specific), if a high percentage of the serotyping results obtained during analysis are in agreement with (i.e. are "concordant" with) the genotype of virus identified by PCR based methods such as RFLP or sequencing.

The level of concordance is calculated by dividing the number of results which were ingreement with the genotype of infecting virus identified by PCR

based methods by the total number of samples which were reactive on the serotyping assay.

$$\text{i.e.} \quad \frac{\text{No. of serotyping results which agree with genotyping analysis}}{\text{No. of samples reactive on the serotyping assay}}$$

(c) Discrepant

A serotyping result is "discrepant" if it is not in agreement with the result obtained using other methods of genotyping.

(d) Non-type-specific (NTS)

Samples showing non-type-specific reactivity are those which show the presence of NS4 antibody (by reactivity in the unblocked well), but the serotype cannot be distinguished by the assay. This kind of reactivity can be observed on the microtitre plate in two ways; (1) The unblocked well is positive with all other wells negative. In these cases, cross reactivity may be taking place between the NS4 antibody in the sample and one or more of the competing peptides in solution. This may occur if a patient is infected with an unusual subtype of HCV, in which NS4 antigenic variation in NS4 has resulted in shared epitopes with those of a different genotype. (2) All eight wells show high levels of reactivity. The antibody titre in these samples may be at too high a level for the 100 fold excess competing peptide solution to absorb all non specific antibody. Clearer results are usually observed in these cases if the sample is retested using a higher dilution of serum.

(e) Non Reactive (NR)

Samples which are unreactive in all eight wells on the assay do not contain antibody to this region of NS4.

2.2 ELISA

OPD substrate was dissolved in citrate phosphate buffer pH 4.0 at a concentration of 0.5mg/ml, with 0.1% hydrogen peroxide (Dako Ltd) being added immediately prior to use.

Citrate phosphate buffer; 0.08M citric acid (SIGMA)
0.1M Na_2HPO_4
Adjust pH to 4.0 with NaOH
Store at 4°C, maximum 4 weeks.

2.3 RESTRICTION ENDONUCLEASES

The restriction sites for the restriction enzymes used in this study are as follows;

Rsa I	GT / AC
Hae III	GG / CC
Mva I	CC / (A,T)GG
Hinf I	G / ANTC
*Bst*U I	CG / CG
ScrF I	CC / NGG

2.5 DNA SEQUENCING

10x Sanger TBE; 162g Tris base (AnalaR, BDH)
42.5g Boric Acid (Scientific Imaging Systems, Eastman Kodak Co., Rochester, NY)
9.5g EDTA (Scientific Imaging Systems)
Made up to 1 litre with distilled water

2.6 CLONING

Luria Broth; 10g tryptone (DIFCO)

5g yeast extract (GIBCO. BRL)

5g NaCl (Fisher scientific)

Made up to 1 litre with distilled water

Adjust pH to 7.0-7.5 with NaOH

Solution III; 29.5ml glacial acetic acid (AnalaR. BDH)

Adjust to pH 4.8 with KOH pellets (AnalaR. BDH)

Made up to 100ml with distilled water

2.7.3 PROTEIN EXPRESSION

SOB Agar; 20g Bacto Tryptone

5g Yeast extract

0.5g NaCl

Add distilled water to 1 litre

Adjust to pH 7.5 with KOH, Autoclave

Add 20ml 1m $MgSO_4$ prior to use.

Lysis buffer 20mM Tris-HCl pH 7.9

0.5M NaCl (5mM imidazole)

Protease inhibitors; Leupeptin, Pepstatin, E64 (all at 1μg/ml)

N.B. No EDTA or DTT must be present if the proteins are to be purified by immobilised metal affinity chromatography, as they will react with the nickel ions in the resin.

2.7.4 SDS-PAGE

15% Resolution Gel

2.35ml water

2.5ml 1.5M Tris pH 8.8

0.1ml 10% SDS

5.0ml Acrylamide / Bisacrylamide

50μl ammonium persulphate

5μl TEMED

15% Stacking Gel

6.1ml water

2.5ml 0.5M Tris pH 6.8

0.1ml 10% SDS

1.3ml Acrylamide / Bisacrylamide

50μl Ammonium persulphate

10μl TEMED

1x SDS-PAGE running buffer

10% Ultra Pure 10x Tris (0.25M) / Glycine (1.92M) / SDS (1%) (National Diagnostics)

90% distilled water

2x SDS sample buffer

25ml 4x Tris-HCl / SDS pH 6.8 (below)

20ml Glycerol

4g SDS

3.1g DTT

1mg Bromophenol Blue

Distilled deionised water to 100ml

4X Tris-HCl / SDS pH 6.8

0.5M Tris-HCl

0.4% SDS

pH 6.8 with 1N HCl

water added to 100ml.

Solution is filter sterilised through a 0.45μm sterile filter.

Coomassie Blue staining solution

50% (v/v) methanol

0.05% (v/v) Coomassie Brilliant blue R-250 (BioRad).

10% (v/v) acetic acid

40% distilled deionised water

Coomassie blue is dissolved in the methanol prior to the addition of the acetic acid or water.

Destaining solution

7% Acetic acid

5% methanol

88% water

2.7.5 WESTERN BLOTTING

1x Transfer buffer

10% Transfer buffer (0.25M Tris, 1.92M Glycine) (National Diagnostics)

19% Methanol

0.04% SDS

70.96% distilled water

10x CAST

800ml distilled water

85g NaCl

75ml 10% Casein solution (Caseign Hamersten; DIFCO)

25ml 2M Tris pH 7.5

10ml Bronidox-L

Adjust to pH 7.8 +/- 0.2 using 5M NaOH or HCl

Add distilled water to 1 litre

2.9 PROTEIN EXPRESSION BY MCAC.

8 X Binding buffer

4M NaCl

160mM Tris-HCl pH 7.9

40mM Imidazole

10mM Imidazole wash

12.5ml 8X Binding buffer

0.5ml 2M Imidazole

Adjust pH to 7.9 with HCl

Make up to 100ml with MilliQ water.

30mM Imidazole wash

As above with 1.5ml 2M Imidazole.

300mM Imidazole wash

As above with 15ml 2M Imidazole.

5.3 PHYLOGENETIC ANALYSIS OF NUCLEOTIDE SEQUENCES

Phylogenetic trees can be either rooted or unrooted. A rooted tree indicates the direction of evolution, with the root representing the common ancestor of all the sequences analysed. An unrooted tree specifies the relationship among the sequences but does not define the evolutionary path.

There are a number of methods avalable for the construction of phylogenetic trees, which are classified into either distance matrix or character based (maximum parsimony) methods. Distance matrix methods involve the calculation of evolutionary distances (pairwise distances) between the sequences, whereas maximum parsimony methods construct a phylogenetic tree by considering the

shortest pathway which leads to certain character states such as nucleotides at a specific site in the sequence.

There are two different types of methods available for testing the reliability of a phylogenetic tree. These are the maximum likelihood method (Felsenstein, 1981) and the bootstrap test (Felsenstein, 1985). The maximum likelihood method examines the reliability of every interior branch of the tree, and is generally time consuming and limited to use with small data sets. The bootstrap involves random resampling of the data from which the tree was constructed, in order to produce another tree. This process is repeated several hundred times and the frequency at which particular branches are encountered in the newly constructed trees is calculated to give a probability for each branch in the original tree. This statistical method is used with both neighbor-joining and maximum parsimony methods.

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Sequence Analysis of Hepatitis C Virus Variants Producing Discrepant Results With Two Different Genotyping Assays

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Methods for identifying the genotype of hepatitis C virus (HCV) in clinical specimens are frequently based upon the direct characterisation of viral RNA sequences by polymerase chain reaction (PCR) amplification, or by serologically based methods, in which the infecting genotype is inferred from the pattern of antibody reactivity to type-specific peptides or recombinant proteins used as antigens in an Enzyme Linked Immunosorbent Assay (ELISA). Although genotyping by direct, PCR-based methods show generally highly concordant results with the genotype inferred from serological typing assays (>95% agreement), there exist a small number of samples that produce discrepant results. To investigate the underlying reasons for the discrepancies, we obtained eleven samples from haemophiliacs and four samples from patients with chronic hepatitis C that produced discordant results between a PCR based assay (InnoLipa I and II) and a serotyping assay (Murex HC02). Nucleotide sequences in the 5′noncoding region (5′NCR), core, and NS4 region were used to identify the genotype of the circulating virus and to identify amino acid changes in NS4 that might alter antigenicity. In 14 samples, sequence analysis of all three regions was concordant with the results of the InnoLipa assay. There were few if any amino acid substitutions in NS4 that might have accounted for the discrepant serotyping results, which were found predominantly in samples from individuals with a history of multiple exposure to HCV. It remains unclear whether the detection of antibody in such discrepant samples corresponds to previous expression of a different genotype than detected by PCR, or whether the virus population in plasma is more restricted in genotype diversity than the population in the liver or at other sites of viral replication. ***J. Med. Virol. 53:237–244, 1997.***

KEY WORDS: serotyping; genotyping; genotype; NS4; 5′ noncoding region

INTRODUCTION

Hepatitis C virus (HCV) is a positive strand RNA virus which was first cloned in 1989 and is the major aetiological agent of non-A non-B hepatitis worldwide [Choo et al., 1989]. The virus genome is approximately 9400 nucleotides in length, and displays up to 30% sequence divergence between each of the six major types, each of which can be further divided into numerous subtypes [Simmonds et al., 1993a; Bukh et al., 1993a; Simmonds et al., 1994a; Mellor et al., 1995a].

Recent investigations have suggested that infections with HCV type 1 are associated with more rapid disease progression and are more likely to cause hepatocellular carcinoma than infection with other genotypes such as types 2 or 3 [Takada et al., 1996; Kobayashi et al., 1996]. Another reason for investigating the infecting HCV genotype in clinical samples is in the evaluation of patients for antiviral treatment, since there is an increased frequency of sustained response to interferon in individuals infected with types 2 and 3, compared to HCV type 1 [reviewed in Davis, 1994, Dusheiko et al., 1994].

Because of these clinical associations, a number of different methods have been developed to identify the virus genotypes in infected individuals. The majority of techniques are based on the amplification of subgenomic fragments of HCV by the polymerase chain reaction (PCR). Genotypes can be distinguished using amplified DNA sequences in three different ways: (1) by differential amplification using type specific primers [Okamoto et al., 1992; Okamoto et al., 1993; Widell et

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al., 1994; Ohno et al., 1997], (2) by the digestion of amplified PCR products from the 5′ noncoding region (5′NCR) with combinations of restriction endonucleases, to reveal patterns of fragments specific to individual genotypes [McOmish et al., 1994; Davidson et al., 1995; Mellor et al., 1996], or (3) by the reverse hybridisation of labelled PCR products to immobilised type specific probes [Stuyver et al., 1993; Stuyver et al., 1995; Stuyver et al., 1996]. A different, indirect method for typing virus in infected individuals is based upon the observation that sequence diversity between genotypes results in the production of type-specific antibodies, which can be detected by ELISA [Machida et al., 1992; Tanaka et al., 1994; Simmonds et al., 1993; Zhang et al., 1995; Dixit et al., 1995; Bhattacherjee et al., 1995]. The usefulness of serotyping assays lies in their rapidity and ease of use, as long as this is not at the expense of sensitivity or accuracy.

In this study, we have compared the performance of one direct (Inno-LiPA I and II) and one indirect (Murex 1-6 serotyping) typing assay on two distinct populations: a group of haemophiliacs from Germany, and patients with chronic hepatitis C from France. Discrepant samples were further investigated by sequence analysis of the 5′NCR, core and NS4 regions.

MATERIALS AND METHODS

Samples

Serum samples were obtained from haemophiliacs in Frankfurt, Germany, and from patients with chronic HCV infection in France. Additional samples were obtained from some individuals with discrepant results in order to permit sequence analysis.

Genotyping

Isolates were genotyped using Inno-LiPA HCV version I or version II (Innogenetics, Antwerp, Belgium) according to the manufacturer's instructions. This assay used labelled PCR products from the 5′NCR as probes for reverse hybridisation to oligonucleotides immobilised on strips [Stuyver et al., 1993; Stuyver et al., 1996].

Serotyping

Type-specific antibody was determined by the Murex 1-6 serotyping assay HC02 (Murex Biotech, Dartford, UK), or where specified, the Murex 1-3 serotyping assay HC01, according to the manufacturer's instructions. Type-specific peptides corresponding to two epitopes in NS4 were used as antigen in a competitive ELISA [Simmonds et al., 1993; Bhattacherjee et al., 1995].

RNA Extraction and Nested Reverse-Transcription PCR (RT-PCR)

Virus RNA was directly extracted from 100 μl serum using proteinase K/sarcosyl and phenol/chloroform extraction. RNA was precipitated at −20°C overnight in ethanol and dissolved in 25 μl DEPC treated water prior to RT-PCR [Davidson et al., 1995].

(1) 5′ NCR. Reverse transcription of RNA used the outer antisense primer 209, followed by primary PCR with primers 209 and 939 (outer sense) and secondary PCR with inner primers 940 (sense) and 211 (antisense) [Chan et al., 1992].

(2) Core. Core specific antisense primer 410 was used for reverse transcription of RNA, followed by PCR amplification with primers 410 and 954 (outer sense), and inner primers 951 (sense) and 953 (antisense) [Mellor et al., 1995].

(3) NS4. NS4 amplification was achieved using combinations of universal primers, and type-specific primers designed for HCV types 1, 2 and 3, as follows; outer antisense primers included 007 [Simmonds et al., 1993b], LP2 (type 1 specific) 5′GTACTGTATCCCGCTGATGAARTTCCACA 3′, and LP4 (type 3 specific) 5′GTATTGGATCCCACTCACAAAATTCCACA 3′. Outer sense primers were 5668 5′ATGCATGTCRGCTGAYCTGGA 3′ and 53510 [Bhattacherjee et al., 1995]. For the second round of PCR, primers were 220 [Simmonds et al., 1993b], 865 [Prescott et al., 1996], LP3 (antisense, type 1) 5′TTCCACATGTGYTTCGCCCAGA 3′, LP6 (sense, type 1) 5′CTGGAGGTCGTCACTAGCACCTGG 3′, LP15 (antisense, type 2) 5′CAAAGCTTANAGCATCTCSGCNATCCGCTGCCC 3′, LP12 (sense, type 2) 5′GCTCTCGAGCCSGACAAGGARRTCYTNTAT 3′, LP5 (antisense, type 3) 5′TTCCACATATGCTTGTGCCAGA 3′ and LP7 (sense, type 3) 5′CTGGAAGTAACCACCAGCACCTGG 3′. PCR products were electrophoresed through a 2% agarose gel containing 0.5 μl/ml ethidium bromide, and viewed on a u.v. transilluminator.

Sequencing

PCR products were either sequenced directly (5′NCR and core regions) or from clones. Direct sequencing involved the re-amplification of the 2° PCR product with one of the primers biotinylated, followed by immobilisation on streptavidin-coated magnetic beads (Dynabeads M280, Dynal) as previously described (Kleter et al., 1994). Sequencing reactions were performed using T7 DNA polymerase (Sequenase version 2.0, United States Biologicals, USB) according to the manufacturer's instructions. Sequences from the 5′NCR, core, and NS4 regions have been submitted to GenBank and bear the accession numbers AF007498-AF007522.

Cloning of NS4

TA cloning of amplified NS4 fragments into the pTAG vector was carried out using the LigATor kit (R&D systems, Europe, Ltd.) according to the manufacturer's instructions. Cloned NS4 fragments were sequenced after the denaturation of miniprep DNA with 2M NaOH/2mM EDTA, followed by precipitation in ethanol at −70°C for 15 minutes. To investigate the possibility of a mixed infection, up to five different clones were sequenced for samples GD1, GD3, GD4, GD5, and GD7. Between five and ten individual clones were sequenced for each PCR product from samples giving positive PCR results with more than one set of

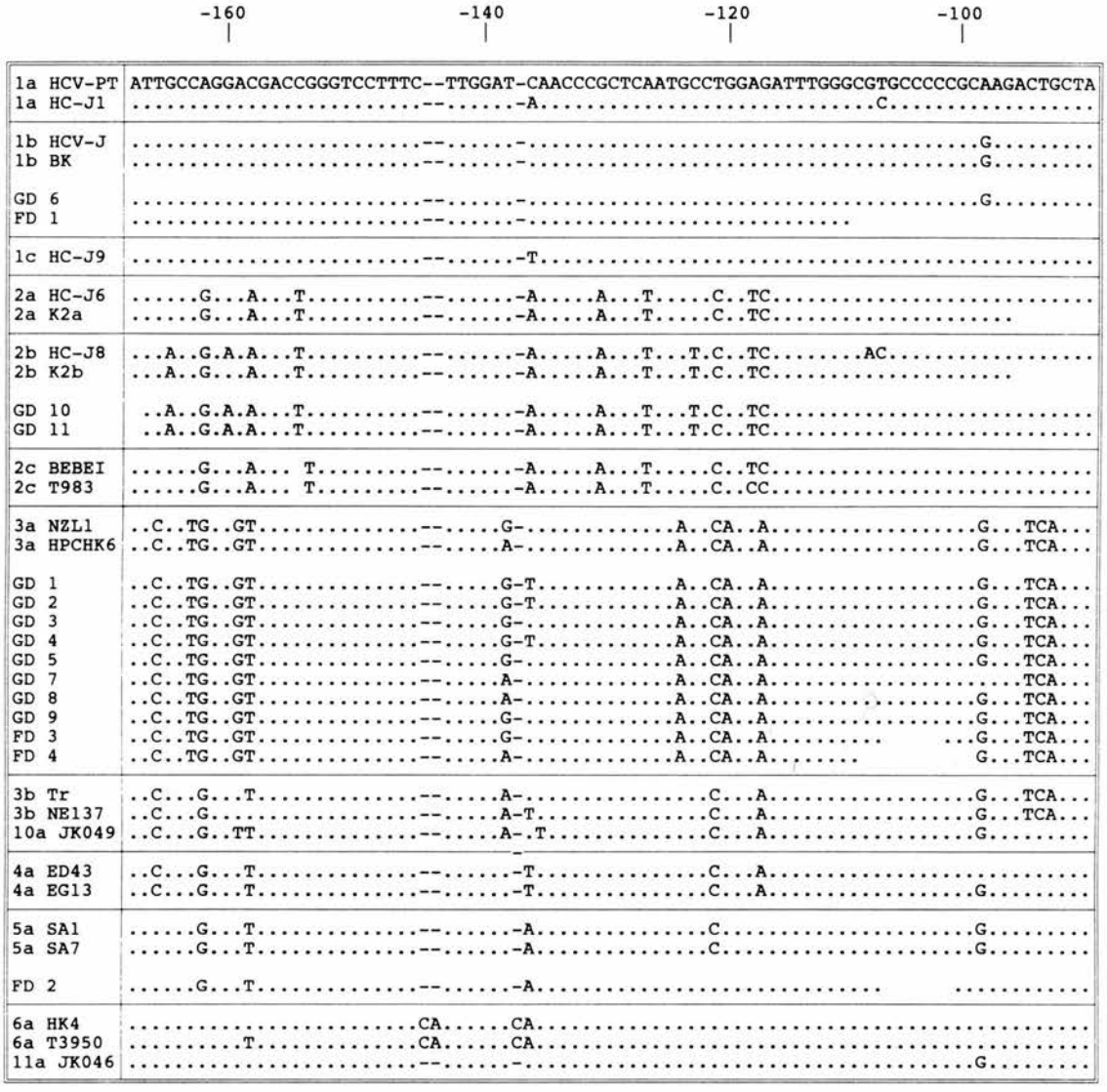

Fig. 1. Comparison of 5′ NCR nucleotide sequences obtained from the discrepant samples with sequences specific for each genotype.

type-specific primers (GD2 and GD8). Sequencing results from the remaining haemophiliac discrepant sera were based on analysis of single clones only.

RESULTS

HCV Positive Haemophiliacs

From a group of 78 HCV PCR positive German haemophiliac samples tested with both the Inno-LiPA II assay and the Murex 1-6 serotyping assay, a total of eleven gave discordant results (Table I). In eight cases the genotype identified in the 5′ NCR was HCV-3a, with type 1 specific antibody being detected by the ELISA. Two samples (GD10 and GD11) were type 2b by Inno-LiPA and type 1 by serotyping, while GD6 was the opposite, being typed as 1b by Inno-LiPA II and as type 2 by serotyping. In sample GD1, types 1 and 2 were detected by serotyping, but the genotype was HCV-3a by Inno-LiPA.

RNA sequences from the eleven samples were amplified by PCR in the 5′ NCR and NS4 regions, and where serum volume permitted, in the core region. The HCV genotype identified in each sample by direct sequence analysis of the 5′NCR [Smith et al., 1995] was concordant with that detected by the Inno-LiPA II assay (Fig. 1), and in each case phylogenetic analysis of sequences in both the core and NS4 regions gave the same genotype assignments as deduced for the 5′NCR. In order to rule out the possibility of a dual infection, PCR amplification of NS4 was carried out using primers specific for the genotypes detected by Inno-LiPA and serotyping (types 1, 2, or 3). In all but two samples, a positive PCR result was identified only when we were using primers specific to the genotype identified by Inno-LiPA (Table I). However, NS4 sequences could be amplified from samples GD2 and GD8 by both type 1 and type 3 specific primers, although the PCR products

TABLE I. Identification of Genotypes in Discrepant Sera Using Different Typing Assays and Sequencing

	Typing results			Type-specific PCR			Sequencing results		
Sample	Inno-LiPA II	Murex 1-3	Murex 1-6	T1	T2	T3	5′NCR	Core	NS4
GD1	3a	—	1 + 2	—		+	3a	—	3a
GD2	3a	—	1	+w		+	3a	3a	3a
GD3	3a	—	1	—		+	3a	3a	3a
GD4	3a	—	1	—		+	3a	3a	3a
GD5	3a	—	1	—		+	3a	—	3a
GD6	1b	—	2	+	—		1b	1b	1b
GD7	3a	—	1	—		+	3a	—	3a
GD8	3a	—	1	+w		+	3a	3a	3a
GD9	3a	—	1	—		—	3a	—	—
GD10	2b	—	1	—	+		2b	2b	2b
GD11	2b	—	1	—	+		2b	2b	—
	Inno-LiPA I								
FD1	1b	NR	4				1b	1b	1b
FD2	4-5	1	1				5	5	5
FD3	3a	NR	1				3a	3a	3a
FD4	3a	NR	1				3a	3a	3a

w = weak result.

with type 1 primers were much weaker under U.V. transillumination than the corresponding bands on the gel using type 3 primers. Multiple clones from each PCR product from GD2 and GD8 were analysed by sequencing, and each was identified as type 3.

One potential explanation for the cases of mistyping by the serotyping assay is that amino acid variation within NS4 in the discrepant samples was sufficient to produce cross-reactivity with peptides normally specific to a different genotype. However, few amino acid differences were observed from the peptides used for serotyping, and were generally restricted to substitutions of residues with similar size and charge (Fig. 2). Although the analysis of multiple clones revealed the same genotype for each isolate, where amino acid sequences from multiple clones show variation, each sequence is shown for comparison.

French Patients With Chronic Hepatitis C

A total of 88 samples from patients with chronic hepatitis C infection in France were analysed using Inno-LiPA I, and the Murex 1-6 serotyping assay. Four samples were discrepant; sample FD1 was typed as HCV-1b by Inno-LiPA I and yet only had evidence of type 4 specific antibody upon testing by the ELISA, FD2 was type 4 or 5 by Inno-LiPA I, but HCV-1 by serotyping, while samples FD3 and FD4 were HCV-3a by Inno-LiPA I but type 1 by serotyping (Table I).

Sequence analysis in 5′NCR of FD1, FD3, and FD4 confirmed the genotype as that identified by Inno-LiPA I, while sample FD2, identified as 4-5 in the 5′NCR by genotyping, was type 5 upon sequencing in all three regions (Fig. 2).

Amino acid substitutions in NS4 were more frequent than in the haemophiliac samples, and occurred in both peptide regions 1 and 2 (Fig. 2). Two amino acid substitutions occurring in region 2 of sample FD1 produced a sequence that resembled that of the peptide of HCV-4a used in the serotyping assay.

DISCUSSION

Genotyping assays are usually based upon the amplification of HCV sequences in the 5′NCR. These allow the identification of each of the six major genotypes but are more limited in their ability to differentiate between subtypes, such as type 1a and 1b, or between type 2a and 2c, which are often identical in this region [Stuyver et al., 1995; Smith et al., 1995]. In addition, the use of genotyping assays presupposes access to PCR technology in the laboratory, and until automation becomes widely available, such methods will be relatively time consuming and expensive. Serotyping assays based on antigenic variation of NS4 or core can distinguish between virus types but are unable to differentiate between subtypes [Machida et al., 1992; Bhattacherjee et al., 1995; Dixit et al., 1995; Zhang et al., 1995]. However, the assays are generally quick and easy to perform and use equipment and techniques that are commonplace in any diagnostic virology laboratory.

A high concordance has been found between the genotypes identified by PCR and the detection of genotype-specific antibody. For example, among reactive samples from Scottish blood donors to branched peptides from NS4, the antibody specificity was 99.2% concordant with the genotypes identified by restriction fragment length polymorphism (RFLP) [Simmonds et al., 1993b]. Similarly, 98.5% of samples were concordant with genotyping by Inno-LiPA I [Lau et al., 1996]. Serologically typeable samples in the modified assay that extended serological detection of types 4, 5, and 6 showed a similar concordance with RFLP (100%, Navas et al., 1997; 98%, Bell et al., 1996; 97%, Bhattacherjee et al., 1995) and other PCR-based typing methods

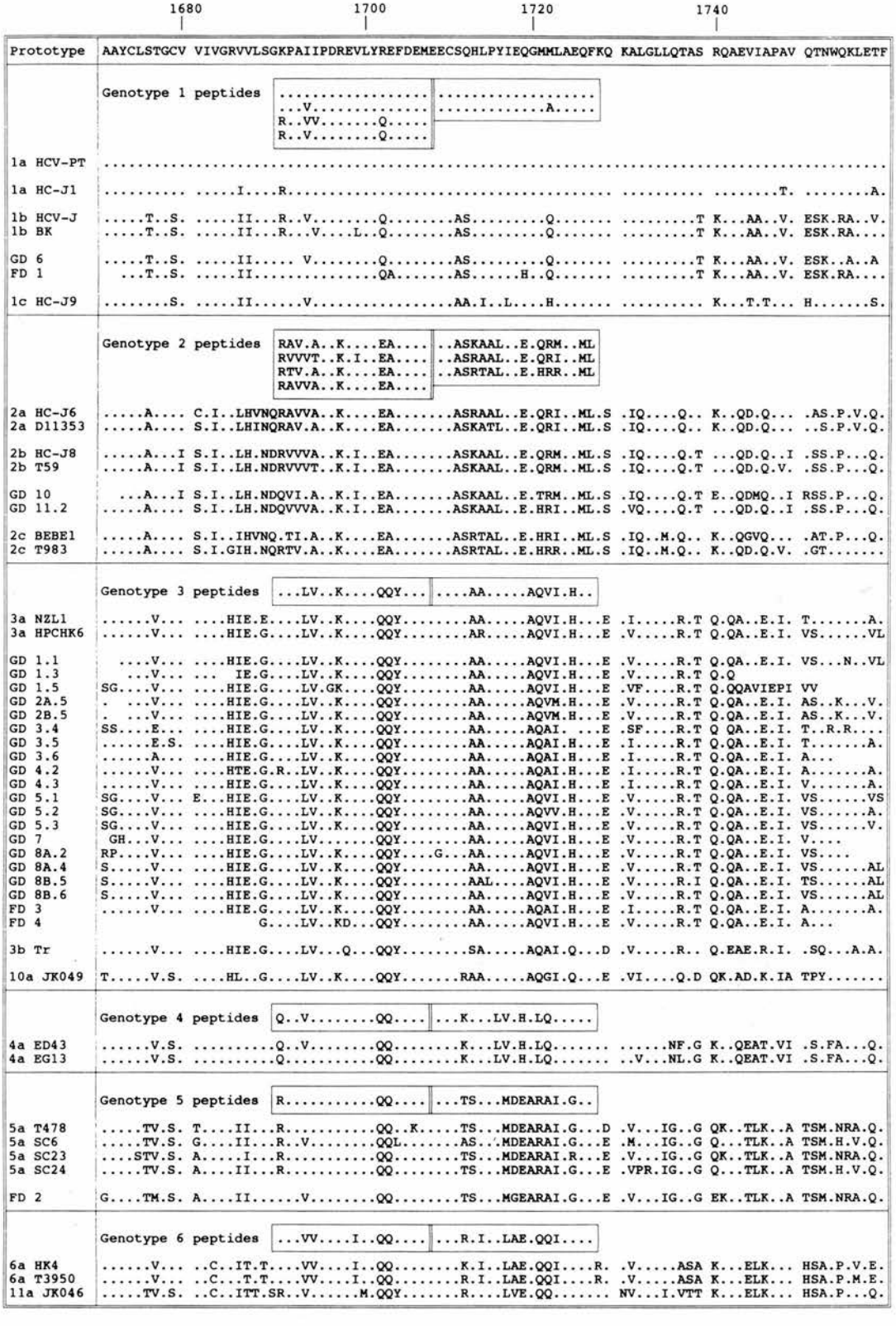

Fig. 2. Comparison of NS4 amino acid sequences obtained from the discrepant samples with those of peptides used in the serotyping assay. Peptides used in the serotyping assay are in boxes. "." indicates sequence identity to HCV-PT. Samples identified with a decimal point represent the reference number of the clone (e.g., GD 3.6 represents clone 6 from GD 3). GD 2A, 8A: sample GD2 and GD8 amplified with type 1 specific primers. GD 2B, 8B: sample GD2 and GD8 amplified with type 3 specific primers.

such as Inno-LiPA, of 94% [Vandoorn et al., 1996]. Other serological typing assays involving the detection of type specific antibody to the core protein have also been shown to be concordant with PCR based typing methods (99%, Dixit et al., 1995 and 100%, Machida et al., 1992), although the genotypes that were detected by these assays was restricted to types 1 and 2.

Discrepancies between the results of serotyping by Inno-LiPA and by the serotyping assay could arise because the serotyping assay detects circulating antibody rather than the infecting virus, and so discrepant results could result from antibody induced by previous infections, especially for samples from individuals with a history of multiple exposure, such as haemophiliacs. Alternatively, the Inno-LiPA and serotyping assays could produce discrepant results if an individual were infected with a recombinant virus that contained 5′NCR and NS4 sequences derived from viruses of different genotypes. Finally, the discordant results could be due to mistyping by one or other of the assays. Unusual nucleotide sequence variation in the 5′ NCR could produce inappropriate hybridisation of genotype-specific probes. Alternatively, amino acid changes in NS4 could influence the adsorption of antibody to peptides normally specific for a heterologous genotype and produce an incorrect result in the serotyping assay.

Multiple Infection

A higher frequency of discrepant results between the two assays was observed in haemophiliacs, who have been multiply exposed to a range of HCV variants (14.1%), than in a patient group where multiple exposure would be expected to be less common (4.5%). Sequence analysis of discrepant samples revealed that the sequence of the 5′NCR was always consistent with the genotype of the predominant circulating variant identified by Inno-LiPA. Furthermore, in all discrepant samples, both the core and NS4 sequences were consistent with the genotype identified by sequencing of the 5′NCR. Type-specific primers in NS4 generally amplified the genotype detected by Inno-LiPA, and in the two cases where amplification was obtained using both the type 1 and 3 primers, only type 3 sequences were detected among the clones derived from the PCR products. Overall, we found no evidence for concurrent infection with multiple genotypes in this risk group. However, there was also no evidence for that the serotyping assay incorrectly identified the specificity of the antibody response since the NS4 amino acid sequence of virus from the discrepant samples corresponded closely to that of the corresponding type-specific peptide in both regions 1 and 2 (Fig. 2). Substitutions occurring in these regions were generally to residues of similar size and charge to those in the peptides (an example was the substitution of lysine to arginine in region 1 of GD 4.2). Another conservative amino acid substitution occurred at position 1723 of type 3a viruses, where valine was often replaced with alanine. Only GD 11 showed an obviously nonconservative amino acid substitution that could affect the antigenicity of NS4a, occurring at the N-terminal end of the region 1 peptide.

For the above reasons, the most likely explanation for the observed discrepant results is multiple infection, particularly in the haemophiliacs who have been exposed to a large number of HCV-contaminated blood products in the past. For example, if a patient initially infected with type 1 was recently reinfected with type 3, only antibody specific for type 1 would be detected within the "window period" for the superinfecting virus, and a discrepant result would be obtained. However, since 1985, all factor VIII and IX concentrates have undergone virus inactivation treatment, so recent de novo infection in this patient group can be ruled out. On the other hand, we have previously found that the major circulating genotype changed in around 30% of haemophiliacs over the seven to ten years during treatment with exclusively heat-inactivated clotting factor. In the absence of re-exposure to HCV, these changes in genotype must have resulted in reactivation rather than reinfection, which indicates that variants of HCV may persist for several years but can remain undetectable by PCR of plasma [Jarvis et al., 1994]. In some haemophiliacs, changes in circulating genotype were followed by corresponding changes in type-specific antibody, whereas in others, no change in antibody specificity developed, and the delays or absence of the appearance of antibody to the new genotype in this study group led to several discrepant results between the serotyping and genotyping assays. Multiple infection is also a possible explanation for the discrepant results observed in FD1, FD2, and FD3, as their risk factors for infection were intravenous drug abuse, an activity likely also to lead to multiple exposure and infection with more than one genotype. Without a comprehensive analysis of genotypes present in the liver or possible extrahepatic sites of HCV replication, we therefore cannot conclude that genotypes detected in plasma are necessarily the only replicating variants of HCV in vivo. Further work characterising the variants in different organs targeted by HCV is clearly necessary to further explore the significance of the discrepant results.

Recombination

There are some reports describing recombinants of HCV [Yun et al., 1996; Kato et al., 1992], although it is difficult to exclude the possibility that these observations resulted from mixed infection, PCR contamination or artifactual recombination during PCR [Meyerhans et al., 1990]. Indeed, a consistent feature of other studies has been that sequence relationships between subgenomic regions always reflect those of the complete genome [Ohba et al., 1995; Smith et al., 1995; Simmonds et al., 1994; Stuyver et al., 1994]. In this study, the same genotype was detected by sequencing 5′NCR, core, and NS4 regions, including the NS4 sequences amplified using type-specific primers. Recombination was therefore not the explanation for any of the discrepant results observed in this study.

Antigenic Variation of NS4

Heterogeneity of the deduced NS4 amino acid sequences in the regions targeted by the serotyping assay was greater in two samples from the French hepatitis C study group than for any from the German haemophiliacs. Sample FD 1 (Inno-LiPA type 1b; serotyping assay type 4) had amino acid substitutions in both peptide regions 1 and 2 (Fig. 1), including the substitution of glutamine for histidine at position 1720. This substitution is also present in the type 4 peptide and is consistent with the observed cross-reactivity in the serotyping assay.

Sequence differences from the type 5a peptide were also observed in sample FD 2 (Inno-LiPA type 4-5, serotyping type 1). In region 1 (positions 1691 and 1694) the sequence of FD2 corresponded closely to one of the type 1 peptides used in the assay (Fig. 2), while in region 2, a substitution of aspartate to glycine (position 1719) was observed, although the sequence remained quite distinct from the region 2 peptides of type 1 (Fig. 2).

In conclusion, the reliability of serological typing methods may be influenced by the patient groups under investigation. Methods based on direct virus detection (such as Inno-LiPA) may be more appropriate for use with patients previously known to have been multiply exposed, such as haemophiliacs, since circulating antibody from previous or multiple infection may occur in these individuals. However, the frequency of discrepant results within samples from patients with chronic disease was much lower, and the simplicity and rapidity of the serotyping assay suggests that it may be a suitable alternative in such study groups.

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Detection and Clinical Features of Hepatitis C Virus Type 6 Infections in Blood Donors From Hong Kong

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The genotype distribution of hepatitis C virus (HCV) was investigated in 212 viraemic blood donors from Hong Kong. A subset of the samples was investigated using three different genotyping assays to establish the accuracy of each in this population. These assays were restriction fragment length polymorphism (RFLP) of amplified 5′ noncoding region (5′NCR) sequences, RFLP of the core region, and a serotyping assay using peptides from two antigenic regions of NS4.

Genotypes detected in Hong Kong blood donors were 1a (6.2%), 1b (58.8%), 2a (1.4%), 2b (1.4%), 3a (1.9%), and 6a (27.0%). All genotyping assays produced concordant results. No evidence was obtained for the presence of type 6 group variants recently identified in Southeast Asia, other than type 6a. A serotyping assay based upon the detection of type-specific antibody to epitopes in NS4 produced similar results to the genotyping assays (98% concordance), but a reduced sensitivity (75%) compared with genotyping methods. Sequence variation in NS4 was not the cause of the reduced rate of detection of type 6 antibody in this population.

Eighty-four percent donors infected with type 6a were male, compared to 75% donors infected with type 1b. The median alanine transaminase (ALT) level in type 6 infected donors was lower than in type 1b, (43.8 and 51.1 U/l, respectively) although these values were not statistically significant ($P = 0.094$). There was no significant difference between the ages of donors infected with types 1b and 6a. Risk factors for HCV infection in the blood donors included blood transfusion, intravenous drug abuse, and tattooing. A significantly greater number of donors infected with HCV-6a reported a history of drug abuse (66%) than donors infected with HCV-1b (7%).

KEY WORDS: HCV, genotypes, serotypes, blood donor, Southeast Asia

INTRODUCTION

Hepatitis C virus (HCV) is the primary aetiological agent of posttransfusion non-A, non-B hepatitis worldwide. The genome comprises single-stranded, positive sense RNA of approximately 9,400 nucleotide bases in length. As with many other RNA viruses, HCV displays extensive sequence variation. At least six major genotypes have been described to date, each of which can be further divided into subtypes according to their degree of sequence similarity to each other, and by their branching order using phylogenetic analysis [Simmonds et al., 1993a, 1994; Bukh et al., 1993]. For example, there is approximately 70% overall sequence identity between the major genotypes and 80% between subtypes. Infections in the United States and western Europe are generally caused by HCV genotypes 1, 2, and 3, although these are also distributed worldwide. Type 4 is found in Central Africa and in the Middle East, while type 5 is common only in South Africa. Of the six major genotypes, type 6a shows one of the most confined geographical locations, having been found only in Hong Kong, Macau, and Vietnam [Mellor et al., 1996; Tokita et al., 1994; Simmonds et al., 1993a], or in emigrants from these countries [Murphy et al., 1994]. Several groups have recently reported the existence of novel genotypes in southeast Asian countries such as Thailand and Vietnam, and have shown these to be closely related to type 6a [Mellor et al., 1996; Tokita et al., 1994, 1995]. Whether or not these are to be classified as separate major genotypes or as further subtypes of HCV-6 has still to be decided [Doi et al., 1996; Mellor et al., 1996].

One potentially important reason for genotype identification is the observation that virus genotype, along with virus load and host-related factors (severity of liver disease), may influence the response to antiviral treatment [Hino et al., 1994; Dusheiko et al., 1994]. Consequently, a number of techniques have been developed to identify HCV genotypes in clinical specimens. The

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majority of these methods use the polymerase chain reaction (PCR), and involve three main procedures to distinguish between genotypes. These are 1) the use of type-specific primers [differential amplification, Okamoto et al., 1992, 1993], 2) the use of type-specific probes to hybridise with amplified HCV DNA after PCR [Stuyver et al., 1993, 1995; Tisminetzky et al., 1994], and 3) digestion of the amplified PCR product using combinations of restriction enzymes [Mellor et al., 1996; Davidson et al., 1995; McOmish et al., 1994]. Serologically based HCV typing methods are competitive or noncompetitive enzyme-linked immunosorbent assays (ELISAs) that detect antibody to type-specific epitopes in the core and NS4 regions [Bhattacherjee et al., 1995; Dixit et al., 1995; Zhang et al., 1995b; Simmonds et al., 1993c; Tanaka et al., 1994; Machida et al., 1992].

Previous studies have compared the accuracy of these different typing methods [Dixit et al., 1995; Lau et al., 1995], but these have not examined more recently discovered variants such as type 6 virus. In this study, we have carried out an extensive survey of samples from Hong Kong blood donors including a large proportion of type 6 infections, and a comparative evaluation of several genotyping assays.

MATERIALS AND METHODS

Samples

A total of 212 serum samples obtained from Hong Kong blood donors (Red Cross Blood Transfusion Service, Yaumatei, Hong Kong) were used in this study. All had previously been identified as either anti-HCV positive or indeterminate using the Abbott Matrix assay. Seventy-four of these samples were used for the comparison of different genotyping assays. Abbott Matrix results, donor sex, age, and serum alanine transaminase (ALT) levels were available for all 212 samples, and risk factors for infection could be identified in 43 cases.

RNA Extraction and Nested Reverse Transcription-PCR (RT-PCR)

Virus RNA was directly extracted from 100 μl serum using proteinase K/sarcosyl and phenol/chloroform. The RNA was precipitated in ethanol at −20°C overnight and dissolved in 25 μl DEPC-treated water prior to RT-PCR [Davidson et al., 1995].

5′ Noncoding region (5′NCR). RNA was reverse transcribed using the 5′ NCR-specific outer antisense primer 209, followed by nested PCR amplification involving outer primers 209 and 939 (sense) and inner primers 211 (antisense) and 940 (sense) [Chan et al., 1992].

Core. Core-specific antisense primer 410 was used for reverse transcription of extracted RNA. Outer primers used for PCR amplification were 410 and 954 (sense), followed by inner primers 953 (antisense) and 951 (sense) [Mellor et al., 1995].

NS4. Based on currently available HCV-6a NS4 sequences [Bhattacherjee et al., 1995], type-specific primers were designed for hemi-nested RT-PCR amplification. Antisense primer 867 was 5′TTCCACATRTGY TTNKSCCAGAA3′, with two sense primers 865 (5′CTGGAGGTTATCACNAGCACNTGG3′-outer) and 866 (5′RTCCTCGTGGGTGGAGTCCTNGC3′-inner)

RFLP Analysis

Amplified DNA was incubated with combinations of restriction endonuclease enzymes overnight, then electrophoresed through a 4% Metaphor agarose gel (containing 0.5 μg/ml ethidium bromide) in 1 × TBE. Restriction patterns of cleaved DNA were viewed on a UV transilluminator.

5′NCR RFLP. All PCR-positive samples were genotyped using a combination of restriction endonucleases *Hae*III/*Rsa*I and *Mva*I/*Hinf*I to distinguish between types 1-6 [McOmish et al., 1994]. As the 5′ NCR of type 6 group variants found in Southeast Asia has previously been shown to produce restriction patterns identical to those of type 1 isolates [Mellor et al., 1996], amplified DNA was also cleaved using enzymes *Dde*I/*Hpa*II, which are able to distinguish some, but not all, of these different variants from type 1. Subtyping of HCV types 1, 2, and 3 was carried out using enzymes *Bst*UI (type 1) and *Scr*FI (types 2 and 3) as previously described [Davidson et al., 1995].

Core RFLP. Samples identified as type 1 or 6 by RFLP in the 5′ NCR were amplified using primers from the core region and cleaved using the restriction endonucleases *Ava*I, *Sma*I, and *Hae*II [Mellor et al., 1996].

1-6 Serotyping Assay

The serotyping ELISA is a competitive assay using type-specific peptides from two antigenic regions of NS4 [Bhattacherjee et al., 1995].

Sequencing

Primary PCR products were reamplified with one of the primers biotinylated and then they were bound onto streptavidin-coated magnetic beads (Dynabeads M280, Dynal). DNA strands were denatured using 0.15 M NaOH and then separated magnetically. The bound strand was sequenced using the "Sequenase" kit version 2.0 (United States Biologicals, USB) according to the manufacturer's instructions.

Statistical and Phylogenetic Analyses

Statistical analysis was carried out using standard statistical software (SYSTAT). Phylogenetic trees were constructed using the PHYLIP program, NEIGHBOR [Felsenstein, 1993].

RESULTS

Comparison of Genotyping Methods

A total of 74 HCV-positive samples were genotyped using three different assays, each based on different regions of the genome. In addition, the majority of the samples were sequenced in the core region to confirm the genotype identification. The first genotyping assay was based on RFLP of amplified sequences from the 5′NCR, and three versions of this assay were used to analyse the samples. The original 5′NCR RFLP used enzymes *Hae*III/*Rsa*I and *Mva*I/*Hinf*I to identify geno-

TABLE I. Identification of Genotypes by RFLP and Sequencing in the 5′NCR, Core, and NS4 Regions

Final designation	No.	5′NCR[a]					Core[a]		Sequencing			
									Core		NS4	
		*Hae*III/*Rsa*I	*Mva*I/*Hinf*I	*Bst*UI	*Scr*FI	*Dde*I/*Hpa*II	*Sma*I	*Ava*I	No.	Type	No.	Type
Type 1a	3	b	A	A	—	A	s1	a1	—	—	—	—
	1	b	A	A	—	A	s5	a1	1	1a	—	—
Type 1b	41	b	A	B	—	A	s1	a3	9	1b	—	—
	1	d	A	B	—	A	s1	a3	1	1b	—	—
	1	e	A	B	—	A	s1	a3	—	—	—	—
Type 2a	3	c	D	—	D	—	—	—	2	2a	—	—
Type 2b	2	d	D	—	F	—	—	—	—	—	—	—
Type 6a	21	h	B	—	—	D	s5	a12	20	6a	11	6a

[a]Restriction patterns classified according to previous analyses: 5′NCR [Davidson et al., 1995]; Core [Mellor et al., 1996].

types 1 through 6 [McOmish et al., 1994]. The other versions used included a modification of the assay involving the use of enzymes *Dde*I/*Hpa*II to identify novel genotypes isolated from South East Asia, and a further assay used to subtype genotypes 1, 2, and 3 [Davidson et al., 1995]. The second genotyping assay was based on RFLP of the core region [Mellor et al., 1995] and was used to distinguish between type 1 and type 6 group variants that may otherwise have produced identical RFLP patterns in the 5′NCR. The third assay was a serotyping assay [Bhattacherjee et al., 1995] which used competitive binding to detect genotype-specific antibody to two antigenic regions in NS4.

The 74 samples were amplified by PCR using primers from the 5′NCR. Amplified DNA was cleaved using *Hae*III/*Rsa*I and *Mva*I/*Hinf*I, allowing all but two of the samples to be provisionally genotyped as types 1, 2, and 6 (Table I). Two samples produced unusual combinations of RFLP patterns (A with *Mva*I/*Hinf*I but d or e with *Hae*III/*Rsa*I) [Smith et al., 1995]. Subtyping was carried out on the type 1 samples using *Bst*UI and indicated type 1a in 4 and type 1b in 43 of the 47 type 1 samples. Three type 2a and two type 2b samples were identified using *Scr*FI.

Restriction analysis with *Dde*I/*Hpa*II revealed that none of the type 1 samples contained substitutions at positions −127 and −155 previously associated with some type 6 group variants [Mellor et al., 1996]. To confirm the genotype identifications of the 5′NCR RFLP assay, all of the type 1 and type 6 samples were amplified in the core region and cleaved with *Sma*I and *Ava*I. These enzymes distinguish type 1a (pattern a1 with *Ava*I) from type 1b (pattern a3) and other type 1 variants (patterns a2,a4) from type 6 and type 6 group variants (patterns a10-a13, sl/s5) [Mellor et al., 1996].

All of the samples identified as type 6 produced the pattern a12s5, confirming their identification. Amongst the four samples identified as type 1a by RFLP in the 5′NCR using *Bst*UI, all produced the a1 pattern. Similarly, all of the type 1b samples identified using *Bst*UI produced the a3 pattern. The two samples with unusual *Hae*III/*Rsa*I patterns (d, e) were typed as 1b using *Ava*I and *Sma*I, and this was confirmed for one of these samples (d) by sequencing in the core region. This analysis failed to reveal any samples of type 6 group variants apart from 6a, and indicates that genotyping in the 5′NCR is accurate in this particular population.

All but two of the samples were assayed for type-specific antibody to NS4 peptides [Bhattacherjee et al., 1995] (Table II). Of those samples with detectable antibody, all but one reacted specifically with the peptides of the genotype identified by RFLP. The only exception was a type 6 sample that reacted with type 1 peptides. This sample was confirmed as type 6 by sequencing in the core and NS4 regions. The reason for the inappropriate activity is unclear. Type-specific antibody was not detected in 18 samples, giving the serotyping assay an overall sensitivity of 75%.

To investigate whether the HCV-6a-specific peptides used in the serotyping assay fully represented the antigenic variability within the genotype, 11 type 6a samples were amplified and sequenced within this region. Results indicate that the type 6 region on which the NS4 peptides were based in the assay was highly conserved (Fig. 1). Variants of type 6a show highly restricted variability through the NS4 region, displaying only two to three nucleotide differences over the 275 bases sequenced. Relatively tight clustering of type 6a variants is observed upon phylogenetic analysis of the NS4 region, compared with the diversity amongst variants of other genotypes such as 1b and 4a (Fig. 2).

Genotype Distribution

HCV RNA was extracted from a further 138 serum samples from Hong Kong blood donors (total 212). Having established the accuracy of the 5′NCR genotyping assay in this population, we used this method (*Hae*III/*Rsa*I and *Mva*I/*Hinf*I followed by *Bst*UI (type 1) or *Scr*FI (types 2, 3) to identify the genotypes present in these samples (Table III). From the 212 positive sera, the two genotypes most frequently detected were types 1b (n = 127, 60%) and 6 (n = 57, 27%). Other genotypes identified were types 1a (n = 13), 2a (n = 8), 2b (n = 3), and 3a (n = 4). A mixed infection was observed in only one donor who was infected with both genotypes 3a and 6a.

Reactivity in the Abbott Matrix Assay

All ELISA positive samples were tested by the Abbott Matrix assay which uses antigens (core, NS3, and NS4)

TABLE II. Detection of Type-Specific Antibody in Samples From Hong Kong Blood Donors

Genotype	Serotype 1	2	6	NTS	NR	Total	Sensitivity (%)	Concordance (%)
Type 1a	3	0	0	0	1	4	75	100
Type 1b	33	0	0	6	4	43	77	100
Type 2a	0	2	0	1	0	3	67	100
Type 2b	0	2	0	0	0	2	100	100
Type 6a	1	0	13	5	1	20[a]	70	93
Total	37	4	13	12	6	72	75	98

[a]Two samples not serotyped due to lack of serum.

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                1680        1690        1700        1710        1720        1730        1740        1750        SERO      O.D.IN WELL
                 ▾           ▾           ▾           ▾           ▾           ▾           ▾           ▾          TYPE     U      1      6
PT      GCVVIVGRVVLSG KPAIIPDREVLYREFDEM E ECSQHLPYIEQGMMLAEQF KQKALGLLQTASRQAEVIAPAVQTNW
HK-4    .....C..IT.T. ...VV....I..QQ.... . ...K.I..LAE.QQI.... R..V.....ASAK...ELK...HSA.
T3950   .....C...T.T. ...VV....I..QQ.... . ...R.I..LAE.QQI.... R..V.....ASAK...ELK...HSA.

HK94          IT.T. ...VV..?.I..QQ.... . ...R.I..LAE.QQI.... R..VF....ASAK...EL          6     >2.0   1.14   >2.0
HK97          IT.T. ...VV..?.I..QQ..?. . ...R.I..LAE.QQI.... R..V?....ASAK...EL          6     >2.0   0.25   0.64
HK99          IT.T. ...VV..?.I..QQ.... . ...RDI..LAE.QQI.... R..V.....ASAK...ELK...      6     >2.0   0.41   1.66
HK103         IT.?. ...VV..?.I..QQ.... . ...R.I..LAE.QQI.... R..V.....ASAK...ELK....S    6     >2.0   0.12   0.89
HK110     .C..IT.T. ...VV..?.I..QQ..?. . ...R.I..LAE.QQI.... R..V...                     6     >2.0   0.18   0.50
HK113  ..E..C..IT.T. ...VV..?.I..QQ..?. . ...R.I..LAE.QQI.... R..V.....ASAK...ELK...HSA. 6     >2.0   0.23   0.52
HK114     .C..IT.T. ...VV..?.I..QQ..?. .   R.I..LAE.QQI.... R..V.....ASAK...           1     >2.0   1.42   0.42
HK157     .C..IT.T. ...IV..?.I..QQ.... . ...R.I..LAE.QQI.... R..V.....ASAK...ELK.       6     >2.0   0.41   0.60
HK77            T. ...VV..?.I..QQ.... . ...R.I..LAE.QQI.... R..V?....ASAK...ELK.       6     1.93   0.09   0.97
HK150     .C..IT.T. ...VV..?.I..QQ.... . ...R.I..LAE.QQI.... R..V.....ASAK...ELK.R.I.   6     1.02   0.02   0.38
HK125         T.TS  ...IV..?.I..QQ.... . ...R.I..LAE.QQI.... R..V.....ASAK...ELK ..HSA. 6     0.57   0.07   0.17
HK185       ..IT.T. ...VV..?.I..QQ..K. . ...R.I..LAE.QQI.... R..V.....ASAK...ELK..                Not done
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Fig. 1. Comparison of HCV-6a amino acid sequences in the NS4 region with the prototype sequence (HCV-PT; Choo et al., 1991) and corresponding serotyping results. Antigenic regions to which serotyping peptides are made are in boxes. Well U = "Unblocked", well 1 = type 1 and well 6 = type 6 specific antibody. (?), sequence not determined. ".": sequence identity to HCV-PT.

derived from type 1a sequences. All 11 sera from individuals infected with type 1a (Table IV) reacted with all antigens of the assay, but 11% of the type 1b sera and 32% of the type 6 sera were unreactive with the NS4 antigen. No significant differences in reactivity were found between genotypes in the core or NS3 proteins.

Epidemiological and Clinical Background of Donors

HCV-positive blood donors were predominantly male (male/female ratio of 3:1; Table III) and this ratio was even higher within the type 6 group (5:1). The sample groups for genotypes 1a, 2a, 2b, and 3a were too small to detect any significant trend in these cases. No significant differences were found in the ages of donors infected with different genotypes (Fig. 3) and the range of ages for infected donors (16 to 59 years) was similar for all genotypes.

Median ALT values for all genotypes fell within the normal range (7-53 U/L) with the exception of type 2b (which are based on very few values) (Fig. 4). Although the ALT values of patients infected with type 6 were slightly lower than those infected with type 1b, this was not found to be statistically significant using Spearmans rank correlation ($P = 0.094$).

Risk factors were obtained from 43 HCV-positive donors. These include intravenous drug abuse (prior to 1984), blood transfusion (prior to 1989), tattooing, and acupuncture. In many cases, donors were identified as having been exposed to more than one risk factor. However, in donors with only one identifiable risk factor, 66% of those infected with HCV-6 had a history of drug abuse, compared to 7% donors with type 1b, while 33% HCV-6 infected donors had been previously transfused compared to 83% of those with type 1b (Table III).

DISCUSSION

The identification of HCV genotypes is important in the investigation of the natural history and clinical outcome of HCV infection. Many different methods which have various advantages and disadvantages, exist to identify genotypes. Although assays should be sensitive and specific, other important considerations include cost, time consumption, and ease of use. In our study, all of the genotyping methods used involved the analysis of amplified HCV cDNA by RFLP, while serotyping was carried out by means of a competitive ELISA using synthetic peptides from two antigenic regions of NS4. Although type 6 group variants, described as types 7–9 and 11 [Tokita et al., 1994, 1995; Mellor et al., 1996], have also been detected in countries close to Hong Kong such as Vietnam, no evidence for these variants was found in the Hong Kong population.

When used on the population of Hong Kong blood

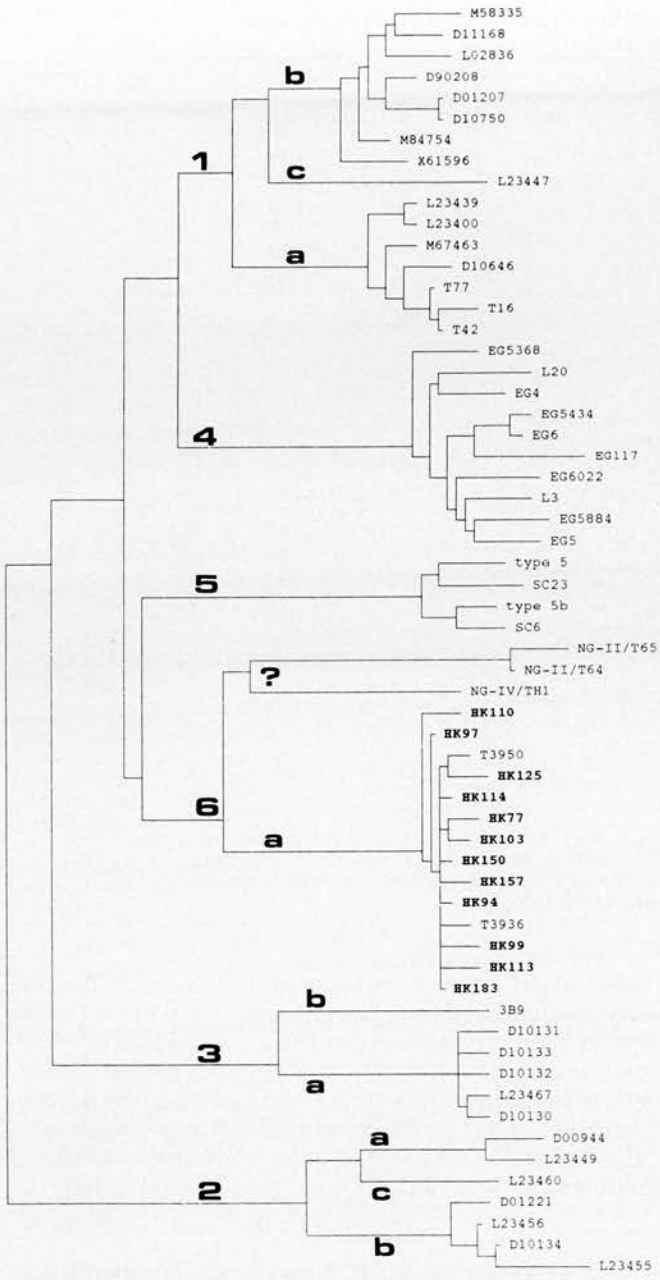

Fig. 2. Phylogenetic analysis of NS4 nucleotide sequences, showing HCV-6a samples from this study in bold.

donors, serotyping using NS4 peptides was found to be 75% sensitive. This low sensitivity may be explained by the observation that detection rates for types 1b and 6 are lower than for other genotypes [Bhattacherjee et al., 1995], at 83% (type 1b; compared to 96% for type 1a) and 67% (type 6). In our study, types 1b and 6 together accounted for 86% of the samples analysed. Higher sensitivities have been reported previously (87–90%) [Zhang et al., 1995b; Dixit et al., 1995; Simmonds et al., 1993c], although in these studies the range of virus genotypes was biased towards types 1a, 2a, 2b, and 3a.

The ELISA could be less sensitive to genotypes 1b and 6a if the peptides used did not fully represent the antigenic variability within those genotypes. However, sequence analysis indicated that this region is highly conserved within most type 6 viruses from Hong Kong (Fig. 1). Another possibility is that certain variants of these genotypes possess sequence changes which result in greater cross reactivity with other genotypes. For example, peptides in antigenic region 1 of type 6 are similar to those of types 1, 4, and 5 [Bhattacherjee et al., 1995]. Further sequence analysis of samples giving nontype-specific results in the NS4 region should reveal the cause of such reactivity.

Within Hong Kong, Macau, and Vietnam, type 6a was responsible for a significant proportion of HCV infections. In this study of HCV infected Hong Kong blood donors, 27% were found to be infected with type 6a. This figure was comparable with that reported from a previous, smaller survey in which HCV-6a accounted for 32% infections in blood donors [McOmish et al., 1994]. In contrast, an investigation of anti-HCV-positive patients in Hong Kong found only 14% to be infected with type 6a [Zhang et al., 1995a]. The fact that fewer patients are infected with type 6a than blood donors suggests that infections with this genotype may have been present in the population for a shorter time period than other genotypes, such as type 1b. Alternatively, it may be that type 6 is less pathogenic than type 1. Differences between genotypes 1 and 2 in their propensity to cause disease have been reported by some [Dusheiko et al., 1994; Booth et al., 1995], but not all investigations [Mahaney et al., 1994; Nousbaum et al., 1995].

Seventy-six percent of HCV-positive blood donors from Hong Kong were male, similar to the relative proportions of male and female subjects donating blood in this population. Similar frequencies of males and females were observed in type 1b infected donors (75%), while a slightly increased proportion of males were infected with type 6a (84%).

A history of drug abuse was reported in 66% of donors infected with HCV-6a, compared to only 7% of donors infected with type 1b. These results differ from a recent survey of HCV infected patients from Hong Kong [Zhang et al., 1995a]. Of eight patients infected with type 6, the source of infection was identified as blood transfusion/renal dialysis in seven and as occupational exposure in the remaining one.

The median ALT level of type 6 infected donors was lower than with type 1b (Fig. 4). Many studies have reported no significant difference in ALT levels associated with infections caused by different genotypes [Dhaliwal et al., 1996; Smith et al., 1996; Dusheiko et al., 1994], although other groups have identified distinct variations among ALT levels, particularly with type 3. However, these findings have been inconsistent, as HCV-3 has been associated with abnormal ALT more often than HCV-1 and HCV-2 [McOmish et al., 1993; Preston et al., 1995], although it also has been connected with having lower ALT values than other genotypes in other studies [Lau et al., 1995].

The future control of HCV infection lies partly with

TABLE III. HCV Genotype Distribution and Clinical Data of Hong Kong Blood Donors

Genotype	No. positive (%)	Donor sex		Mean age (range)	Risk factors				
		Male (%)	Female (%)		Transfusion	IVDA[a]	Tattoo	Acupuncture	None
Type 1	137 (65.0)	103 (76)	34 (24)	34 (16–59)	16	3	5	1	9
HCV-1a	13 (6.2)	10 (77)	3 (23)	35 (16–47)	1	1	0	0	0
HCV-1b	124 (58.8)	93 (75)	31 (25)	33 (17–59)	15	2	5	1	9
Type 2	11 (5.2)	5 (45)	6 (55)	34 (25–48)	0	0	2	0	0
HCV-2a	8 (3.8)	2 (25)	6 (75)	31 (25–48)	0	0	2	0	0
HCV-2b	3 (1.4)	3 (100)	0 (0)	37 (34–39)	0	0	0	0	0
HCV-3a	4 (1.9)	4 (100)	0 (0)	35 (24–42)	1	2	1	0	0
HCV-6a	57 (27.0)	48 (84)	10 (16)	34 (16–52)	3	6	4	0	0
Mixed	1 (0.5)	1 (100)	0 (0)	n/a	0	1	1	0	0

[a]Intravenous drug abuse.

TABLE IV. Reactivity of Blood Donor Samples in Abbott Matrix Assay According to Genotype

Genotype	Total no. samples	Number of reactive samples to Matrix antigens (%)				
		All antigens	Core	NS3	NS4 (yeast)	NS4 (*E. coli*)
1a	13	13 (100)	13 (100)	0 (0)	0 (0)	0 (0)
1b	125	110 (88)	124 (99)	0 (0)	111 (89)	116 (93)
2a	8	3 (38)	8 (100)	0 (0)	3 (38)	4 (50)
2b	3	2 (67)	3 (100)	0 (0)	2 (66)	2 (66)
3a	4	2 (50)	4 (100)	0 (0)	2 (50)	2 (50)
6a	57	38 (67)	57 (100)	1 (2)	39 (68)	42 (74)

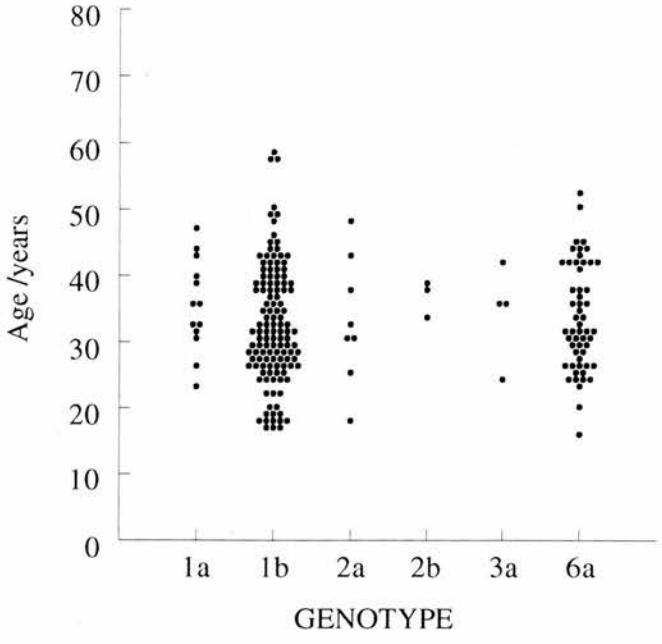

Fig. 3. Age distribution of blood donors infected with different genotypes in Hong Kong.

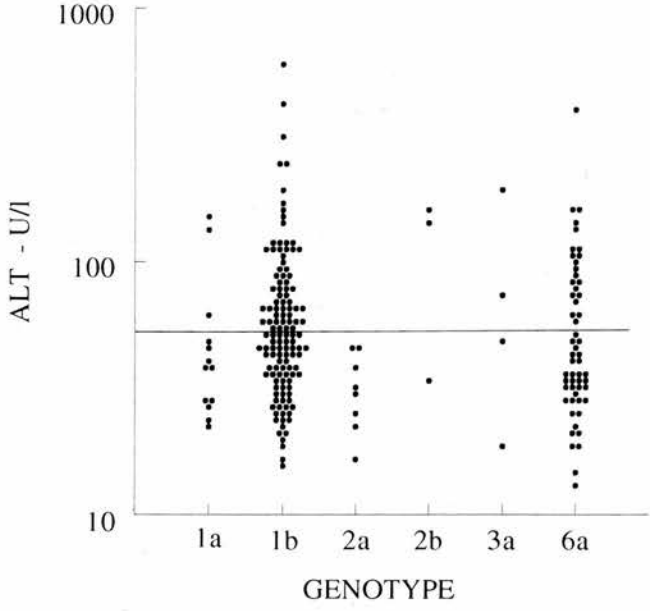

Fig. 4. Comparison of ALT values of donors infected with different genotypes in Hong Kong. Line indicates upper normal range (U/l).

the successful screening and detection of the virus among the blood donor population. Screening assays today use antigens based on sequences from a type 1a prototype virus, and a major concern is that these assays are less effective in the detection of nontype 1 infections [Dhaliwal et al., 1996]. The Abbott Matrix assay was used in the initial screening of these samples, and a lack of sensitivity to type 6 was noted, with 32% being nonreactive to the NS4 antigen. The lack of reactivity to RIBA antigens 5-1-1 and c100-3 by divergent genotypes has previously been reported in other studies [McOmish et al., 1994], and gives rise to concerns that in countries where genotypes other than 1, 2, and 3 exist, routine screening of blood donors could be less effective. One possible safeguard against this would be to include antigens specific to genotypes known to exist in that

area. For example, a screening assay including antigens specific to type 6 as well as type 1 could be used for routine screening in Hong Kong, Macau, and Vietnam.

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Antigenic Variation of Core, NS3, and NS5 Proteins among Genotypes of Hepatitis C Virus

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Assays that detect antibody to hepatitis C virus (HCV) are used to screen blood donors and patients with hepatitis. Current enzyme-linked immunosorbent assay (ELISA)-based methods are invariably based upon antigens from expressed recombinant proteins or oligopeptides from HCV type 1. Some HCV antigens used in screening assays are coded by regions of the HCV genome that show extensive variability; therefore, HCV type 1-based assays may be less effective for the detection of antibody elicited by infection with other genotypes. In this study, we have measured antibody reactivity of sera from 110 hepatitis C patients infected with type 1b, 3a, or 4a to genotype-specific and cross-reactive epitopes present in recombinant proteins from HCV genotypes 1b (core, NS3, and NS5), 3a (NS3, NS5), and 4a (core, NS3), corresponding to those used in current third-generation screening ELISAs. By comparing the serological reactivities of sera to type-homologous and type-heterologous antigens, we detected a significant type-specific component to the reactivity to NS3 (61 to 77% of the total reactivity) and NS5 (60% of the total reactivity). Furthermore, despite the similarities in the amino acid sequences of the core antigens of type 1b and type 4a, we also found significantly greater reactivity to type-homologous antigens, with approximately 25% of reactivity being type specific. These findings are consistent with previous findings of fivefold weaker reactivity of sera from HCV type 2- and HCV type 3-infected blood donors in the currently used third-generation ELISAs and suggest that these assays are suboptimal for screening populations in which the predominant genotype is not type 1.

Detection of antibody to hepatitis C virus (HCV) has become the principal method for the diagnosis of HCV infection in individuals with chronic hepatitis and for the screening of blood donors. Although the original assay based upon the c100-3 recombinant proteins derived from NS4 showed nonspecificity and insensitivity, the more recently developed assays that use recombinant proteins from the core and NS3 regions of the HCV genome (second-generation assays) and the NS5 region of the HCV genome (third-generation assays) have proved to be more effective for the screening of blood donors. Their use has led to a substantial reduction in the incidence of posttransfusion hepatitis. In prospective studies, the incidence of HCV transmission among recipients of blood screened by first-generation assays was 1.5% in Spain (15), 3.7% in Japan (27), and 11% in Taiwan (6). Screening by second-generation assays reduced or would have reduced the incidence to 0.9% in Japan (27), 1% in Spain (15), 2% in Greece (20), and 2.5% in Taiwan (6).

One reason for the failure to detect antibody to HCV in donated blood specimens that transmit HCV infection is that the blood was collected from an individual with acute infection before seroconversion for antibody. This so-called "window" period is long for HCV compared with other viruses for which blood from donors is screened, such as human immunodeficiency virus, with means of 88 to 66 days in second- and third-generation assays, respectively (9). By measuring the incidence of HCV infection in blood donors, it has been estimated that the current residual risk of HCV transmission through collection of "window" samples is 1 per 100,000 donations in the United States (24). This frequency of infection is similar to the residual risk of infection from blood screened by third-generation assays from French blood donors (10).

In addition, samples false negative for antibody have been reported for a small proportion of immunocompetent individuals persistently infected with HCV (2, 17). Indeed, even in anti-HCV-positive individuals, there is considerable variability in the frequency of reactivity to the individual HCV proteins used in supplementary assays, such as the Ortho 3rd generation recombinant immunoblot assay (RIBA-3), and in the titer of antibody to HCV among persistently infected individuals. Among 90 anti-HCV-positive blood donors, antibody reactivity in the Ortho third-generation screening assay varied over a range of 5,000-fold, with some serum samples having antibody levels only just above the cutoff for the assay (12).

HCV can be classified into at least six major genotypes, whose nucleotide and inferred amino acid sequences over the whole genome differ by approximately 30%. This degree of amino acid sequence variability is similar to that observed between variants of other RNA viruses (e.g., dengue virus types 1 to 4), in which significant antigenic differences have been documented and which form the basis of their classification into serotypes. We have previously measured the serological reactivities of individuals infected with different HCV genotypes to antigens used in two third-generation anti-HCV assays (Ortho 3rd generation anti-HCV EIA; Murex VK48).

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Samples from HCV type 1-infected individuals showed an approximately five times greater reactivity than those infected with HCV type 2 or 3, and the reactivity was independent of other factors that may have influenced the antibody response, such as the degree of viremia, donor age, and severity of hepatitis as assessed by alanine aminotransferase measurements.

In the current study we have addressed two of the main shortcomings of the first study. First, we have measured serological reactivities to the individual component antigens used in a third-generation assay (core, NS3, and NS5). These measurements extend previous investigations of type-specific and cross-reactive serological reactivity to the NS4 antigen used in the first-generation screening assays and in second- and third-generation confirmatory assays (1, 5, 14, 21, 26, 28). Second, we have carried out titrations with antigens derived from HCV type 1 as well as corresponding proteins from other HCV genotypes (types 3a and 4a). This allows levels of antibodies to both type-homologous and type-heterologous antigens to be measured, allowing for a more rigorous assessment of the relative levels of type-specific and type-common reactivity to each.

MATERIALS AND METHODS

Samples. Samples LJ516, EG21, and ED43 were obtained from individuals infected with genotype 3a (LJ516) or 4a (EG21 and ED43 [3, 25]). Sequences amplified from the core, NS3, and NS5 regions of types 3a and 4a were used for the synthesis of antigens for antibody screening. Recombinant antigens from the core (amino acid positions 1 to 140), NS3 (amino acid positions 1360 to 1454), and NS5 (amino acid positions 2234 to 2318) (numbered according to Choo et al. [7]) regions of HCV-UK (type 1b) were derived from an existing, commercially available anti-HCV assay (VK48).

Serum samples were obtained from 110 anti-HCV-positive individuals with chronic hepatitis C attending hospital liver clinics in Edinburgh and London, United Kingdom; Karachi, Pakistan; and Cairo, Egypt. Among the samples, we selected 33 infected with genotype 1b, 34 infected with genotype 3a, and 43 infected with genotype 4a. Genotypes were identified by restriction fragment length polymorphism analysis of the 5′ noncoding region as described previously (11).

Development of enzyme-linked immunosorbent assays (ELISAs) based upon type 3a and 4a antigens. RNAs from samples LJ516 (type 3a), EG21 (type 4a), and ED43 (type 4a) were extracted as described previously (16): Reverse transcription was carried out with avian myelobastosis virus reverse transcriptase and RNasin (both from Promega, Southampton, United Kingdom), in each case by using the external antisense primer, and incubation at 42°C for 30 min as described previously (4). The sequences were amplified by using the following heat cycle: 94°C for 18 s, 45°C for 21 s, and 72°C for 90 s for 30 cycles, followed by 6 min at 72°C with the primers listed in Table 1. The core region of type 4a was amplified with primers 954 and 410, followed by amplification with primers D236 and 597. The NS3 region was amplified with primers 751 and 753, followed by amplification with primers 594 and 593 (type 3a) or 750 and 007 and then primers 594 and 593 (type 4a). For the type 4a NS3 region, a third PCR was carried out with the product of the second PCR by using the same inner primers. The NS5 region of genotype 3a was amplified by using primers 991 and 993, followed by amplification with primers 3155 and 3156. The amplified sequences were cloned and sequenced as described previously (23).

Expression of recombinant proteins. The sequences of the core, NS3, and NS5 regions from the different HCV genotypes indicated above were subcloned into *Escherichia coli* or baculovirus expression vectors, as appropriate, by standard molecular biology techniques. The NS3 and NS5 sequences of each genotype were cloned into the *E. coli* vector pTrcHis (Invitrogen BV, Leek, The Netherlands) and were expressed in TOP10 cells by standard isopropyl-β-D-thiogalactopyranoside induction. Core sequences were cloned into the baculovirus expression vector pBlueBacHis2 (Invitrogen), and recombinant virus was derived by cotransfection into insect cells along with linear *Antographa californica* nuclear polyhedrosis virus DNA. Recombinant antigens were produced in Sf.9 insect cells in an LH Series 210 bioreactor by infection at multiplicity of infection of 2 for 48 h.

Both the *E. coli* and baculovirus vector expression vectors place a tract of six histidine residues at the amino terminus of each recombinant protein. Recombinant proteins were therefore subsequently prepared by metal chelate chromatography on Probond nickel affinity resin (Invitrogen) to approximately 90% purity.

ELISA for detection of antibody to core, NS3, and NS5 regions. To optimize plate coating, purified antigens were titrated to levels giving acceptable specificity and sensitivity and approximately equivalent amounts of protein per well (25 to 50 ng). Binding of antigen was assessed both by functional enzyme immunoassay (EIA) with positive and negative sera and by probing for the histidine tag present on each protein by using horseradish peroxidase chemically modified to bind nickel ions. All antigens were coated overnight in 50 mM Tris (pH 8.5) containing 0.02% sodium dodecyl sulfate. The plates were then blocked by the addition of 2% degraded gelatin and were finally dried to ensure stability.

TABLE 1. Primers used for amplification of core, NS3, and NS5 regions

Primer no.	Sequence[a]
954	ACT GCC TGA TAG GGT GCT TGC GAG
410	GAG CGG AAT GTA CCC CAT GAG GTC GGC
D236	GGT AGA CCG GAT CCC ATG AGC ACG AAT CCT AAA
597	GAA AGA AGC TTA GAC GAG CGG AAT GTA CCC
751	TTY CGG GCN GCY GTG TGC ACC
753	GCG TCA TAG CAC TCA CAG AGG AC
594	ACC RCT GCA GAY CCY AAC ATN GAG GAR GT
593	CAC AAG CTT ARC AGT AGT CKA TSA CNG ART C
750	TGG TYA CNN GNN AYG CYG ATG TCA TYC C
007	ACC TCG AGT ATC CCA CTG ATG AAG TTC CAC AT
991	TGG CNA GCT CNT CNG CNA GCC A
993	GGC TCC CCC TCA AGG GRG GCA TRG A
3155	GGA TCC AAY ATN ACY CGY GTR GAG TCW SA
3156	AAG CTT GTG GTG GYA AGG CAC AYC C

[a] International Union of Pure and Applied Chemistry ambiguity codes were used: Y, C/T; R, A/G; N, G/C/A/T; K, G/T; S, C/G; W, A/T. Cleavage sties for restriction enzymes are underlined: CTGCAG, *Pst*I; AAGCTT, *Hin*dIII; GGATCC, *Bam*HI.

For each antigen assay, each serum sample was diluted 1:10, 1:40, 1:160, and 1:640 in anti-HCV-negative sera. Aliquots of 20 μl from each titration were added to 180 μl of sample diluent, mixed, and then added to a well of the ELISA plate. A solution of 1:50 *E. coli* blocker was added to the diluent for all assays for NS3 genotype 3a antigen. The plates were then incubated at 37°C for 1 h, followed by five washes in glycerate-borate buffer. Bound antibody was detected by the addition of 100 μl of horseradish peroxidase-labelled antibody to human immunoglobulin G conjugate and by incubation for 30 min at 37°C. After washing, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate was added, and the mixture was incubated for 30 min at 37°C. The color development reaction was stopped by the addition of 50 μl of 2 M H_2SO_4, and optical densities (ODs) were measured at 470 nm. All assay plates included a dilution series of the reference genotype 1a sample (dilutions of 1:2 to 1:2,560) for calculation of antibody levels. The NS3 genotype 3a recombinant antigens were expressed in *E. coli* and required the addition of 1:50 *E. coli* blocking solution in the sample diluent for all assays.

Quantification of antibody levels. Antibody reactivity to each recombinant protein in each of the test serum samples was determined by titration and reference to a standard positive control as described previously (12). This method is based upon the observation of a linear relationship between OD (over the range of values of 0.02 to 1.0) and concentration of antibody in both ELISAs based upon single antigens, as in the current study and in the currently used third-generation screening assays from Ortho and Murex (12). Because of this relationship, the antibody reactivity of a test sample can be expressed relative to that of a reference control by the following formula: test antibody level = (test OD × reference concentration)/(reference OD × test concentration). Antibody levels were obtained for each sample in each antigen assay in this way.

Estimation of relative proportions of type-specific and cross-reactive antibodies. The ratio of antibody levels measured to type-homologous and type-heterologous antigens indicates the relative proportions of type-specific and cross-reactive reactivities. This calculation is complicated by the possible existence of differences in the antigenicity or presentation of proteins from different genotypes in the ELISA and differences in antibody levels elicited by infection with different genotypes.

The proportion of antibody reactivity that is cross-reactive between genotypes is measured by reciprocal assays of reactivity between sera and antigens from two genotypes, genotypes A and B. In the following relations, E_{BA} represents the antibody reactivity of a serum sample infected with genotype B with antigen of genotype A, A_{AB} is the overall antigenicity of the genotype A antigen relative to that of the genotype B antigen, and T_c and T_s are the proportions of the antibody response that are type common and type specific, respectively, such that $T_c + T_s = 1$.

$$\frac{E_{BB}}{E_{BA}} = \frac{E_{BB}}{E_{BB} \times A_{AB} \times T_c} = \frac{1}{A_{AB} \times T_c}$$

$$\frac{E_{AB}}{E_{AA}} = \frac{E_{AA} \times (1/A_{AB}) \times T_c}{E_{AA}} = \frac{T_c}{A_{AB}}$$

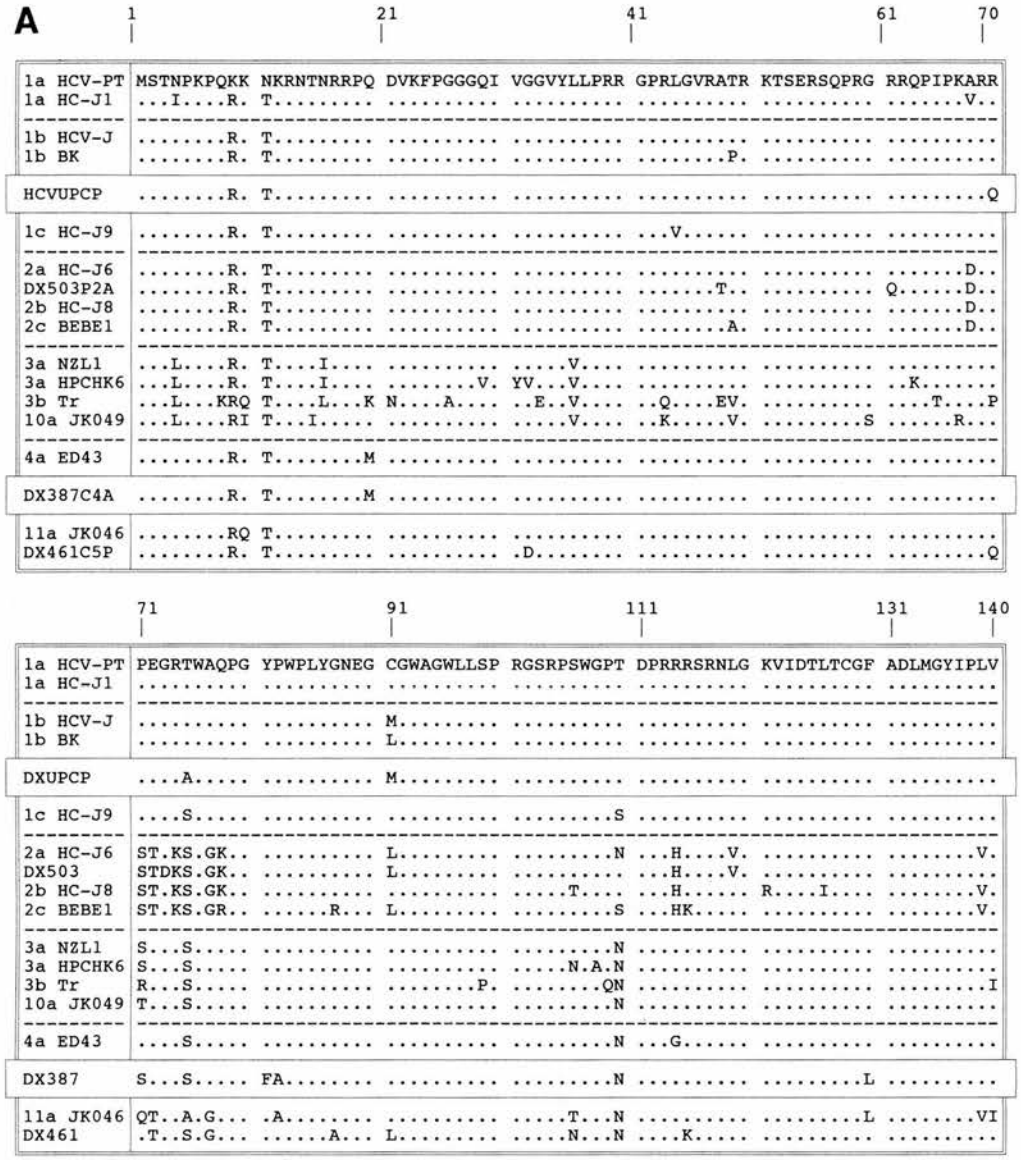

FIG. 1. Comparison of the inferred amino acid sequences of antigens expressed from recombinant clones of (A) core (HCVUPCP, type 1b; DX387, type 4a), (B) NS3 (HCVUPCP, type 1b; DX506, type 3a; DX499, type 4a), and (C) NS5 regions (HCVUPCP, type 1b; DX507, type 3a) with representatives of each of the major HCV genotypes and subtypes. Periods indicate identity with the HCV prototype sequence, HCV-PT (7). Numbers refer to amino acid positions on HCV-PT.

Therefore,

$$\frac{E_{BB}/E_{BA}}{E_{AB}/E_{AA}} = \frac{A_{AB}}{A_{AB} \times (T_c)^2} = \frac{1}{(T_c)^2}$$

From this the proportion of type-common reactivity can be derived as follows:

$$T_c = \sqrt{\frac{E_{AB}/E_{AA}}{E_{BB}/E_{BA}}}$$

and

$$A_{AB} = \sqrt{\frac{1}{(E_{AA}/E_{AB}) \times (E_{BA}/E_{BB})}}$$

These relations make the assumption that the relative proportions of type-specific to type-common reactivity in a type A antiserum are the same as those found in a type B antiserum.

Nucleotide sequence accession numbers. The sequences obtained in the course of this project have been submitted to GenBank and bear the following accession numbers: DX387, AFO29298; DX506, AFO29297; DX499, AFO29296; DX507, AFO29299.

RESULTS

Variability of core, NS3, and NS5 region sequences. The amino acid sequences of the recombinant proteins used in the ELISA were deduced from the nucleotide sequences of the corresponding clones and were compared with other published sequences of these regions of HCV (Fig. 1). In the core protein only 9 amino acids differed between genotypes 1b and 4a over the 140-amino-acid length (6% divergence). Substitutions were generally conservative, where only one resulted in a change in the ionic charge. The NS3 sequences exhibited more variability, with the sequence of genotype 1b differing from that of genotype 3a by 15 of 95 amino acids (16% divergence) and from that of genotype 4a by 13 amino acids, while genotypes 3a

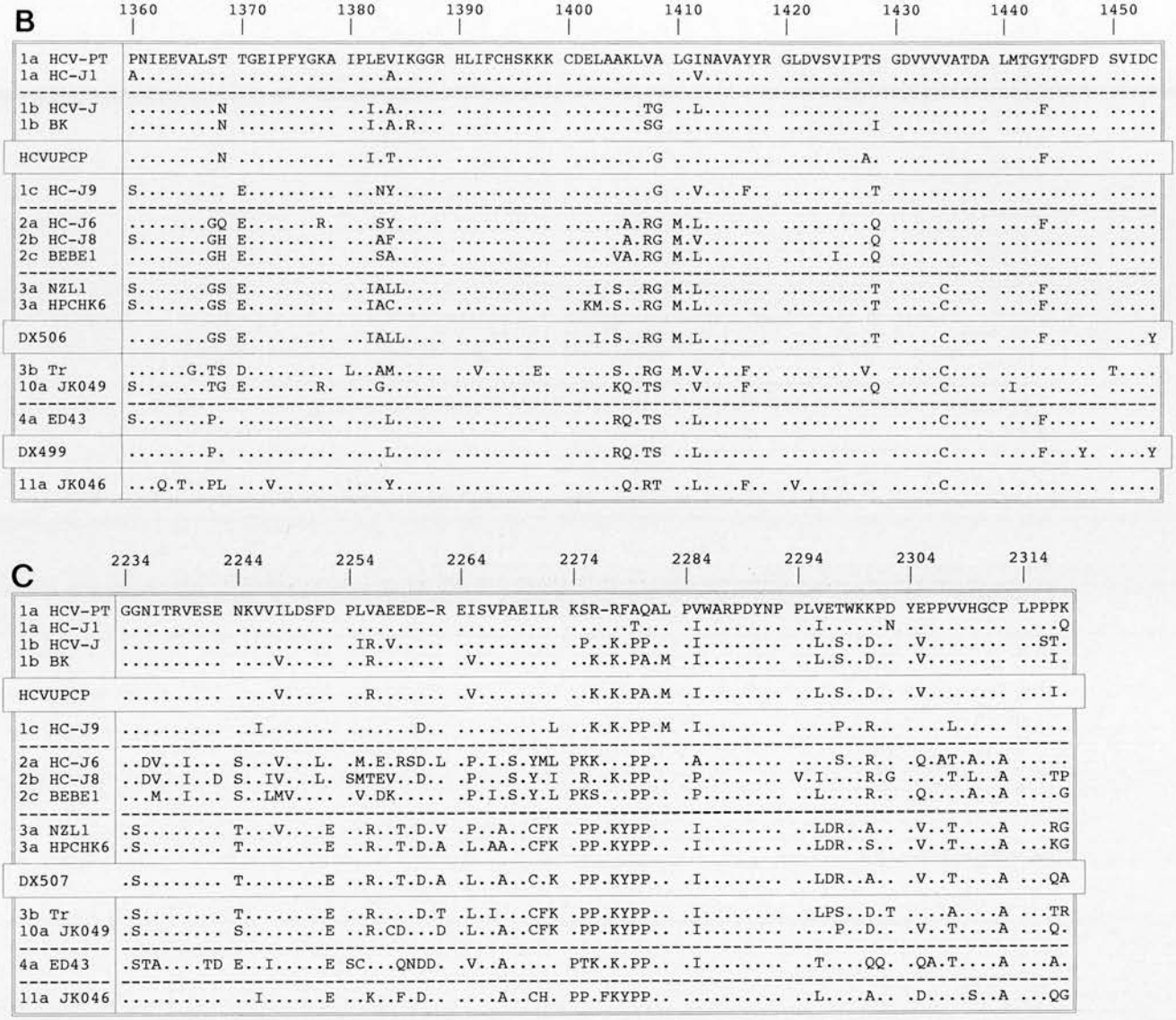

FIG. 1—*Continued.*

and 4a differed from each other by 14 amino acids. In the NS5 sequence there were 23 differences between genotypes 1b and 3a over a length of 85 amino acids (27% divergence). Six of these substitutions affected the charge of the protein: four substitutions of nonpolar amino acids for basic groups and two substitutions of acidic groups for nonpolar groups.

Quantitation of antibody reactivity to core, NS3, and NS5 antigens. The serological reactivities of sera from 33 individuals with genotype 1b, 34 individuals with genotype 3a, and 43 individuals with genotype 4a HCV infections to each of the recombinant antigens from genotypes 1b, 3a, and 4a were measured. To investigate the reproducibility of the method used to quantify antibody levels, each of the serum specimens was assayed in replicate against the type 1b and 4a core proteins (Fig. 2). A close correlation was observed between the two measured antibody levels, with nonparametric correlation coefficients being 0.877, 0.889, and 0.862 for type 1b, 3a, and 4a antisera, respectively. For the type 4a core antigens, the corresponding correlation coefficients were 0.784, 0.721, and 0.802.

There was also a close correlation between reactivity to the core protein of type 1b and that to type 4a (Table 2), with correlation coefficients of 0.834, 0.765, and 0.767 for type 1b, 3a, and 4a antisera, respectively. Significant correlations were also consistently observed between the reactivities of antisera to NS3 proteins of types 1b, 3a, and 4a, as well as between the reactivities of antisera to NS5 proteins of type 1b and 3a. In contrast, there was little if any correlation between antibody reactivity to different regions of the genome. For example, the reactivity of sera from type 1b-infected individuals to the type 1b core protein showed no correlation with reactivity to type 1b NS3 or NS5 proteins (correlation coefficients, −0.118 and −0.181, respectively; Table 2). The only exceptions were weak correlations (0.443 and 0.471) between the reactivities to 3a NS3 and NS5 (but only for type 1b sera) and between NS5 of type 1b with NS3 of 4a (restricted to type 3a sera).

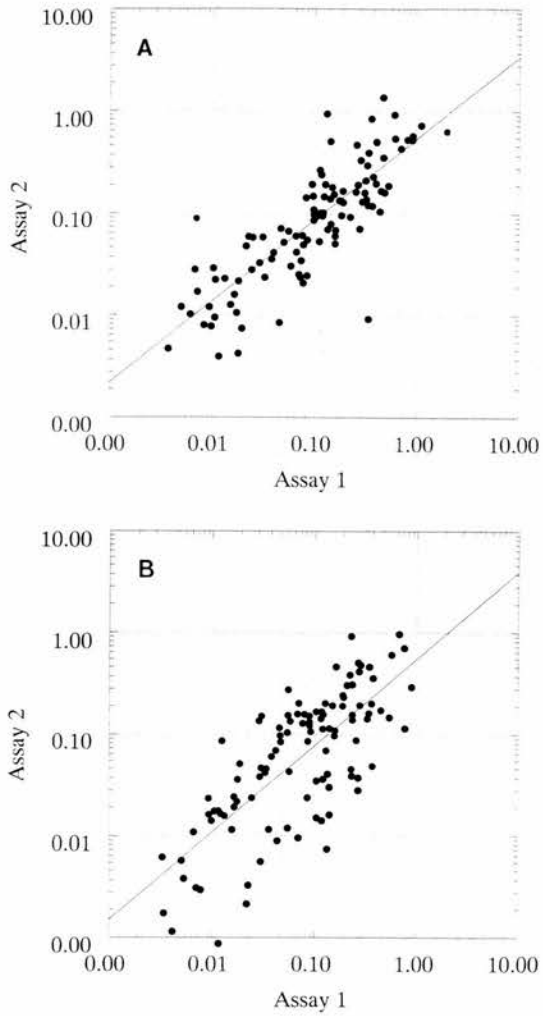

FIG. 2. Measurement of the reproducibility of measurement of levels of antibody to type 1b (A) and type 4a (B) core protein by sera from study subjects by repeat testing. The correlation is indicated by a regression line.

Genotype dependence of serological reactivity. Sera collected from individuals infected with different genotypes varied in their frequency of reactivity to NS3 and NS5 antigens of different genotypes (Table 3). For NS3, frequencies of reactivity for type 1b, 3a, and 4a antisera to type-homologous NS3 proteins were 85, 100, and 76%, respectively, compared with a range of 44 to 76% for type-heterologous combinations. Similarly, the frequency of reactivity of type 1b sera with type 1b NS5 antigen (65%) was higher than that of heterologous sera (47 and 51%), as was reactivity to type 3a NS5 (58% type-homologous reactivity, compared with 24 and 45% for type-heterologous combinations). In the core region, high frequencies of reactivity were observed for both type-homologous (95 to 97%) and type-heterologous (86 to 97%) combinations, indicating the greater antigenicity of this region of the genome and/or a greater proportion of shared epitopes between genotypes.

To compare the strength of reactivity with type-homologous and type-heterologous antigens, antibody levels relative to those for the positive control calculated from OD readings at dilutions ranging from 1:10 to 1:640 were used to calculate antibody levels relative to those for the positive control (Fig. 3). There were significant differences in antibody reactivity to the type 1b core protein between samples from individuals infected with genotype 1b and those from individuals infected with genotype 4a ($P = 0.033$) but not those with genotype 3a ($P = 0.811$). The distribution of antibody levels directed to the genotype 4a core antigen showed no significant difference between genotypes.

Antibody reactivity against NS3 was frequently undetectable among sera from individuals infected with HCV with type-heterologous antigen (Table 3; Fig. 3C). The distribution of levels of antibody to type-homologous antigens was consistently greater than those to type-heterologous antigens. For example, the median level of antibody to type 1b NS3 in type 1b sera was 0.225, which was substantially greater than the median reactivities of type 3a and 4a sera to this protein (0.003 [$P = 0.004$] and 0.02 [$P = 0.005$], respectively). Similar, predominant type-specific reactivities against type 3a and 4a antigens were observed (Fig. 3D and E).

Only 54% of the samples reacted with the NS5 genotype 1b antigen and 43% reacted with the genotype 3a antigen. The median antibody level of genotype 1b sera (0.031) was eightfold higher than that for type 3a samples (0.004) and threefold higher than that for type 4a (0.012). The distribution of antibody levels among genotype 1b samples was significantly higher than that among genotype 4a samples ($P = 0.029$) but not genotype 3a samples. The reactivity of type 3a sera against

TABLE 2. Correlation between reactivity to different antigens

Antigen and genotype	HCV genotype in serum	Correlation for the following antigens and genotypes[a]:						
		Core		NS3			NS5	
		1b	4a	1b	3a	4a	1b	3a
Core								
1b	1b	(1)						
	3a	(1)						
	4a	(1)						
4a	1b	0.834	(1)					
	3a	0.765	(1)					
	4a	0.767	(1)					
NS3								
1b	1b	−0.118	0.023	(1)				
	3a	0.133	0.279	(1)				
	4a	0.100	−0.035	(1)				
3a	1b	−0.270	−0.066	0.456	(1)			
	3a	0.153	0.274	0.363	(1)			
	4a	0.159	0.030	0.627	(1)			
4a	1b	0.048	0.224	0.710	0.518	(1)		
	3a	0.215	0.130	0.570	0.393	(1)		
	4a	−0.058	−0.087	0.497	0.272	(1)		
NS5								
1b	1b	−0.181	−0.220	0.150	0.275	0.157	(1)	
	3a	0.066	0.000	0.218	0.265	0.471	(1)	
	4a	0.058	−0.192	0.069	0.168	0.055	(1)	
3a	1b	−0.141	−0.43	0.247	0.443	0.348	0.630	(1)
	3a	0.051	0.172	0.216	0.266	0.202	0.649	(1)
	4a	−0.076	−0.108	0.186	0.179	0.041	0.303	(1)

[a] Correlation is indicated by Spearman's rank correlation coefficient. Separate values are provided for antisera of each genotype. Significant values ($P < 0.05$) are underlined.

TABLE 3. Frequency of reactivity to core, NS3, and NS5 proteins

Antiserum	No. of specimens reactive to the following antigen/total no. of specimens (%):						
	Core		NS3			NS5	
	1b	4a	1b	3a	4a	1b	3a
Type 1b	32/33 (97)	32/33 (97)	28/33 (85)	25/33 (76)	24/32 (76)	21/33 (64)	8/33 (24)
Type 3a	33/34 (97)	30/34 (88)	15/34 (44)	33/33 (100)	9/33 (27)	16/34 (47)	18/31 (58)
Type 4a	37/43 (86)	41/43 (95)	31/43 (72)	27/43 (63)	31/41 (76)	22/43 (51)	19/42 (45)

NS3 of genotype 3a was significantly greater than that of the type 1b or 4a sera ($P = 0.002$ and 0.01, respectively).

Ratio of type-specific and cross-reactive reactivities. The ability to measure the reactivity of the same antiserum to antigens of different genotypes allowed for an estimation of the relative levels of genotype-specific and cross-reactive serological reactivities. For the core proteins, the levels of antibody to the genotype 1b antigen in each of the type 1b and 4a sera were

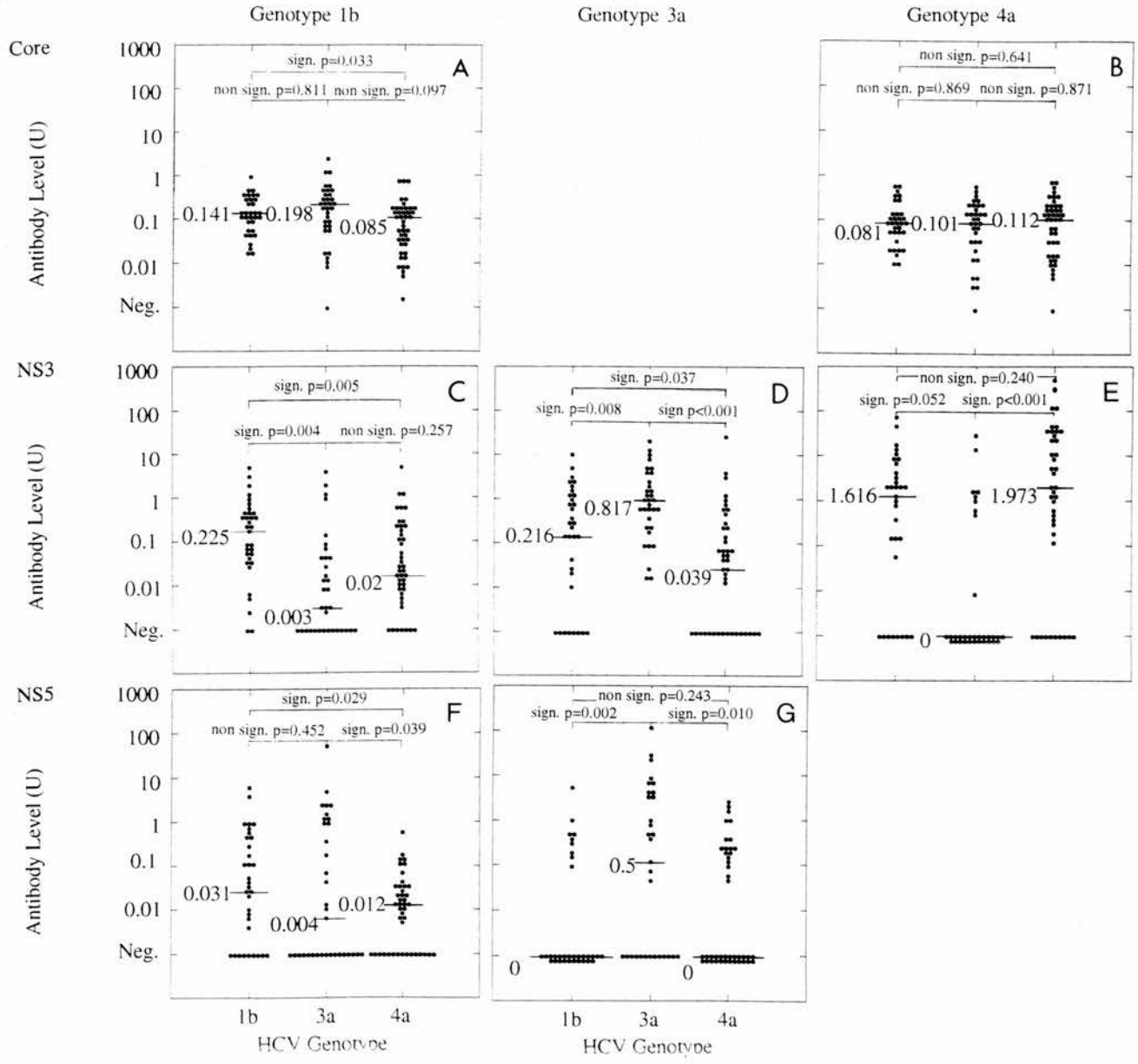

FIG. 3. Distribution of antibody reactivity of sera from individuals infected with type 1b, 3a, and 4a to core proteins of genotypes 1b and 4a (A and B, respectively); NS3 of genotypes 1b, 3a, and 4a (C, D, and E respectively); and NS5 of types 1b and 3a (F and G, respectively). Median values are indicated by short horizontal bars. Pairwise comparison of the distributions of values was carried out by the nonparametric Kruskall-Wallis test.

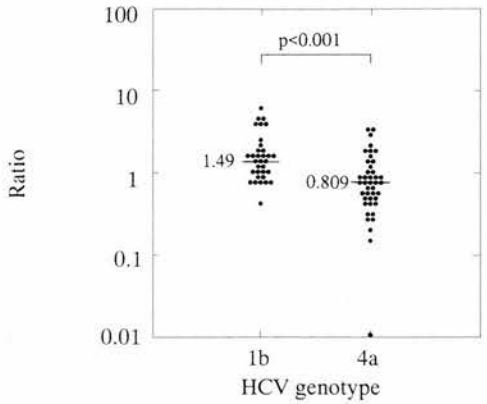

FIG. 4. Ratios of reactivity of type 1b and 4a sera to type 1b/type 4a core antigens. Median ratios are indicated by short horizontal bars. Pairwise comparison of the distributions of values was carried out by the Kruskall-Wallis test. Ratios of reactivities to other antigens are presented in Table 4.

divided by their levels to the genotype 4a antigen (Fig. 4; Table 4). The median ratio of reactivity to type 1b/reactivity to type 4a antigens for type 1b antisera was 1.49, compared with a median ratio of 0.809 for the type 4a antisera. This indicates that type 1b sera react more strongly against the type 1b (homologous) core protein, while the type 4a sera react slightly more strongly against the type 4a antigen. If it were shown that both core proteins were coated equivalently on the solid phase and showed equal antigenicity, then the type-specific reactivity to these proteins could be calculated from the difference from the ratio of 1 that would be expected from exclusively type-common reactivity. However, it is possible that some antigens are present at higher available concentrations than others through differences in binding to the solid phase or solubility. These differences were taken into account by using the derivation described in Materials and Methods. In this instance, the term E_{AB}/E_{AA} represents the median of the ratio of reactivity between type 4a antisera with type 1b and type 4a antigens (0.809). The term E_{BB}/E_{BA} is similarly represented by the median ratio of the type 1b antisera (1.49). Therefore, the proportion of type-common reactivity can be calculated as 0.74, with type-specific reactivity forming the remainder of the reactivity (0.26).

The median ratio of reactivity of the type 3a sera to type 3a and 1b NS5 antigens (0.654) was substantially lower than that of type 1b sera (median, 4; Table 4). Therefore, the proportions of type-common and type-specific reactivity were 40 and 60%, respectively; i.e., the majority of the serological reactivity to this antigen was type specific. Finally, three sets of pairwise comparisons can be made for the NS3 region, in which antigens for all three genotypes were available. Sixty-two percent of the serological reactivity between type 1b and 3a proteins was type specific, similar to the proportions of 61% between type 1b and 4a proteins and 77% between type 3a and 4a proteins (Table 4).

DISCUSSION

Antigenic variability of HCV. The aim of this study was to investigate the degree of type-specific serological reactivity to antigens used in current, third-generation screening assays. The use of recombinant antigens expressed from different genotypes in the enzyme immunoassay allowed reciprocal measurements of type-homologous and type-heterologous reactivity to be made, and these provided a more rigorous assessment of the type-specific components of reactivity to the core, NS3, and NS5 regions. This addresses a potential criticism of previous investigations that showed weaker reactivity of sera from individuals infected with non-type 1 genotypes in either of the screening ELISAs or to individual antigens in the confirmatory recombinant immunoblot assay (5, 8, 12, 14, 21, 28), in that there is a possibility that these observations resulted from a generally weaker serological response to infection than that elicited by type 1. In the current study we were able to consistently show stronger reactivity of sera to antigens of a homologous type than to antigens of heterologous types.

From pairwise reciprocal measurements of antibody reactivity (such as type 1b and 4a antisera against type 1b and 4a antigen), it was possible to quantify the relative contributions of type-specific and cross-reactive antibody reactivities by using the relation derived in Materials and Methods (Table 4). These calculations were independent of possible differences in the strength of the serological response elicited by infection with different genotypes and were also independent of differences in antigen concentration or overall antigenicity between proteins of different genotypes (expressed as the ratio A_{AB}). This removes the potential criticism that the coating efficiency of antigens from different genotypes onto the solid phase was not compared prior to measurement of antibody levels. From this analysis we found a relationship between the degree of amino acid sequence divergence between recombinant proteins and their degree of cross-reactivity. The sequence of the core protein was the most conserved and the core protein showed approximately 25% type-specific reactivity, while the more divergent sequences, those of the NS3 and NS5 regions, showed substantially greater proportions of type-specific reactivity. These results are consistent with previous comparisons of the type-specific component of reactivity to peptides corresponding to linear epitopes in NS4. By absorption in solution with peptides of heterologous genotypes, it was shown that reactivity to type-homologous peptides was reduced but was rarely eliminated (1, 26), allowing development of a sensitive and specific serological typing assay. The type-specific component of serological reactivity to NS4 and, in some studies, to the core protein has been also used in typing assays without cross-absorption, because it has consistently been observed that reactivity to type-homologous antigens in these (13, 19, 22) and other regions (29) is stronger.

Implications for screening assays. The effect of the demonstrated antigenic variabilities of the components of third-gen-

TABLE 4. Type-specific and type-common serological reactivity to HCV core, NS3, and NS5 antigens

Region	Antiserum		Median ratio[a]		P value[b]	Percent		
	A	B	A	B		T_c	T_s	Amino aid divergence[c]
Core	4a	1b	0.809	1.49	<0.001	74	26	6.4
NS5	3a	1b	0.654	4.0	0.014	40	60	27
NS3	3a	1b	0.053	0.373	0.001	38	62	16
	4a	1b	0.016	0.107	<0.001	39	61	14
	4a	3a	0.049	0.939	<0.001	23	77	15

[a] Ratio of reactivity to antigens of genotypes A and B.
[b] Comparison of the distribution of ratios of reactivity of individual antisera to antigens of genotypes A and B by the Kruskall-Wallis nonparametric test.
[c] Degree of amino acid sequence divergence between antigens of genotypes A and B.

eration screening assays on their overall sensitivity for screening is difficult to estimate with precision for two reasons. Individuals vary in their serological responses to different antigens, and therefore in the extent to which these responses may result in cross-reactivity with antigens of heterologous genotypes. For example, if reactivity were directed solely to the core protein, this protein would cross-react with antigens of heterologous genotypes, whereas sera monoreactive with NS3, such as is found upon seroconversion, would be expected to be predominantly type specific.

Furthermore, each of the three antigens investigated is likely to contain a range of linear and conformational epitopes, and these will vary in their degree of cross-reactivity. Recognition of different epitopes in the antigens may be one explanation for the wide range of ratios of reactivity to type-homologous and type-heterologous antigens observed between sera of the same genotype (Fig. 4). In the extreme case, it is possible that reactivity confined to epitopes in the core protein that are type specific would lead to poor or absent reactivity to the core protein of other genotypes. Conversely, the high degree of cross-reactivity observed between certain sera with NS3 or NS5 proteins of heterologous genotypes may have resulted from their recognition of shared epitopes.

The observed differences in reactivity of sera to homologous and heterologous proteins would only lead to false-negative results on serological screening if antibody levels in samples to be tested were close to the cutoff sensitivity of the assay. In a previous study, we measured antibody reactivity in the Ortho third-generation assay and found a wide range of antibody levels (approximately 5,000-fold) among samples from blood donors infected with genotypes 1, 2, and 3, with the lowest level found to be 0.0007, which is just above the cutoff value of the test (12). Low antibody levels and reactivity to a restricted range of epitopes, such as those in NS3, are found in acutely infected individuals (18), and it is likely that earlier detection of seroconversions, and therefore a reduction in the window period associated with non-type 1 infection, may be achieved by assays containing NS3 and other antigens from a wider range of genotypes.

The finding of significant antigenic variability of antigens used for serological screening will form the basis for a number of future investigations. Now that antigens from other genotypes have been produced, it will be possible to carry out large-scale screening of populations infected with non-type 1 genotypes (for example, with the type 4a antigens for testing individuals in the Middle East). This may reveal the frequency with which anti-HCV samples are being missed by conventional assays and may ultimately help reduce the frequency of posttransfusion hepatitis further, particularly when populations with a high frequency of acute infection are screened. Similarly, the incorporation of antigens from other genotypes (particularly NS3) in confirmatory assays may resolve the results for a number of the indeterminate samples identified in blood donor screening and for which interpretation of results is currently problematic.

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Survey of Type 6 Group Variants of Hepatitis C Virus in Southeast Asia by Using a Core-Based Genotyping Assay

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Previous surveys of the prevalences of genotypes of hepatitis C virus (HCV) in different populations have often used genotyping assays based upon analysis of amplified sequences from the 5′ noncoding region (5′NCR), such as restriction fragment length polymorphism (RFLP) or hybridization with type-specific probes (e.g., InnoLipa). Although highly conserved, this region contains several type-specific nucleotide polymorphisms that allow major genotypes 1 to 6 to be reliably identified. Recently, however, novel HCV variants found in Vietnam and Thailand that are distantly related to the type 6a genotype (type 6 group) by phylogenetic analysis of coding regions of the genome often have sequences in the 5′NCR that are similar or identical to those of type 1 and could therefore not be identified by any assay of sequences in this region. We developed a new genotyping assay based upon RFLP of sequences amplified from the more variable core region to investigate their distribution elsewhere in southeast (SE) Asia. Among 108 samples from blood donors in seven areas that were identified as type 1 by RFLP in the 5′NCR, type 6 group variants were found in Thailand (7 from 28 samples originally identified as type 1) and Burma (Myanmar) (1 of 3) but were not found in Hong Kong ($n = 43$), Macau ($n = 8$), Taiwan ($n = 6$), Singapore ($n = 2$), or Malaysia ($n = 18$). Although this small survey suggests a relatively limited distribution for type 6 group variants in SE Asia, larger studies will be required to explore their distribution in other geographical regions and the extent to which their presence would limit the practical usefulness of 5′NCR-based genotyping assays for clinical or epidemiological purposes.

Hepatitis C virus (HCV) is a positive-stranded RNA virus of approximately 9,400 nucleotides and has been shown to be the major etiologic agent of parentally transmitted non-A, non-B hepatitis (7). Sequence comparisons of variants from different geographical areas have led to the identification and classification of at least six major genotypes, many of which contain a number of more closely related, yet distinct subtypes of the virus (5, 20, 24). Phylogenetic analysis of complete genomic sequences (16) or even relatively short subgenomic regions (core [6, 15, 24], E1 [5], NS-4 [2, 21], or NS-5 [11]) may be used for virus classification into genotypes. The overall sequence similarities over complete genomic sequences are at least 91% within variants of the same genotype, approximately 79% (range, 77 to 80%) between subtypes, and about 68% (range, 66 to 69%) between different types (26).

Infection with different genotypes may produce clinically relevant differences in the liver disease caused by HCV. For example, there are now several reports describing a greater sensitivity of HCV genotypes 2 and 3 to interferon treatment than is found in type 1-infected patients (for recent reviews, see references 4 and 19). Assays that can identify the infecting genotype without having to sequence the virus directly are therefore becoming important in patient management and in epidemiological studies of HCV transmission. Published genotyping methods fall into one of two categories, those based upon direct virus detection by reverse transcription-PCR, followed by analysis of the amplified DNA, or indirect (serological) assays, in which the infecting genotype is inferred from the pattern of antibody reactivity to type-specific epitopes in the core or NS-4 region (2, 14, 21, 25).

Although the 5′ noncoding region (5′NCR) of HCV is highly conserved between genotypes, it is an attractive target for genotyping assays, because of the strong association of specific nucleotide polymorphisms with genotype (for a recent review, see reference 22). These genotypes may be readily identified by restriction endonucleases or by type-specific probes (9, 27), although the accuracy for the detection of genotypes 1 to 6 is limited to 96 and 84% for the two assays, respectively (22), mainly because of misidentification of more recently described genotypes.

Recently, several new variants have been identified among blood donors and hepatitis C patients in Vietnam (26) and Thailand (1, 15). Sequence comparisons of the core, NS-5, and E1 regions indicated that these new genotypes often showed sufficient nucleotide differences from existing variants to be classified as new major genotypes. However, we have found that these novel variants group with type 6 upon phylogenetic analysis in both the E1 and NS-5 regions (15), producing what may equally well be interpreted as a single, highly diverse single genotype that includes the previously described type 6a

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genotype found in Hong Kong and Macau (5, 9, 20). These new variants are referred to as type 6 group variants in this study.

Although type 6a and all of the previously described major genotypes (types 2 to 5) show conserved nucleotide differences from type 1 in the 5'NCR that allow them to be differentiated by restriction endonucleases or by type-specific probes in InnoLipa, many (but not all) of the novel variants from Vietnam and Thailand have sequences in the amplified region used for genotyping that are identical to those of type 1a or 1b. Although mixed infection could lead to the detection of sequences of different genotypes in different amplified regions, this explanation was ruled out by finding continuous clones that contained type 6 group sequences in the core and E1 regions but a 5'NCR sequence similar to that in type 1 in several samples from Vietnam (26). This finding leads to new difficulties when interpreting results from genotyping assays based upon the 5'NCR; samples that appear to be type 1 in the restriction fragment length polymorphism (RFLP) assay (9) or InnoLipa (9) may instead have been variants within the type 6 phylogenetic group.

The core gene encodes a putative nucleocapsid protein, which is the most conserved protein in the HCV genome. Nevertheless, it is considerably more variable than the 5'NCR and contains sufficient sequence information to identify all known subtypes and major genotypes, including those in the type 6 group that are misclassified by using the 5'NCR (15). To investigate the distribution of these novel sequences in Southeast (SE) Asia, we have developed a genotyping assay in which sequences in the core region are amplified and cleaved by restriction enzymes that reliably differentiate type 1 variants from type 6 variants. These results provided information on the distribution of type 6 variants in SE Asia and the frequency with which conventional genotyping assays produced incorrect results in different countries.

MATERIALS AND METHODS

Samples. Plasma samples that had been previously genotyped in the 5'NCR by RFLP (9) from blood donors in Macau, Malaysia, Singapore, Taiwan, and Thailand were used in the current study. Additional samples from Hong Kong (n = 66), Malaysia (n = 8), and Burma (Myanmar) (n = 8) were also obtained and genotyped by the same method prior to analysis in the core region. From the blood donor population in Hong Kong, genotypes 1a (Myanmar) (n = 3), 1b (n = 40), 2 (n = 1), and 6a (n = 22) were present, similar to the results of our previous survey (9). From Malaysia, genotypes 1a (n = 10), 1b (n = 8), and 3 (n = 9) were found upon combining the original and new samples. From Burma, types 1b (n = 3), 2 (n = 2), and 3 (n = 3) were identified.

The survey group in the current study was confined to samples of genotype 1 in the 5'NCR RFLP assay that could be amplified in the core region (see below). From Thailand, it was possible to amplify 28 of the 34 type 1 samples in the core region. All of the samples from Burma (n = 3), Hong Kong (n = 43), and Malaysia (n = 18) could be amplified. Lower frequencies of amplification in the core region were obtained from the survey countries surveyed: Macau, 8 of 13; Taiwan, 6 of 53; and Singapore, 2 of 5. Although the core primers are similar in sensitivity to those in the 5'NCR, it is likely that storage conditions influenced the effectiveness of the PCR for the amplification of samples from some countries. A total of 108 samples from all of the countries were used. Previously published sequences from Vietnam and Thailand (1, 6, 26), as well as 25 new core sequences for type 6a (17a), were also included in the analysis.

RNA extraction and nested PCR amplification. Virus RNA was extracted directly from 100 μl of serum with proteinase K-sarcosyl; this was followed by phenol-chloroform extraction and precipitation in ethanol as previously described (9). RNA was reverse transcribed by using the specific outer antisense core primer 410. This was followed by the first round of PCR with primers 410 and 954 and a second round with primers 951 and 953, described previously (15), to give a 405-bp product between positions −21 and +383; the numbering of nucleotides is from reference 8. Sequences in the 5'NCR between positions −245 and −72 were obtained by using primers 209, 939, 211, and 940 (9). The NS-5 region was amplified by using primers 1204, 1203, 518, 517, and 123, as described by Mellor et al. (15).

RFLP analysis. Cleavage reactions were performed on the reverse transcription-PCR 5'NCR product as described previously (9). The restriction enzymes *Hpa*II and *Dde*I identified nucleotide sequence differences in the 5'NCR between type 1 variants and some type 6 group variants (see Results). Restriction endonuclease cleavage of amplified core sequences was carried out by using the restriction endonucleases *Ava*I and *Sma*I. DNA was electrophoresed through a 4% Metaphor agarose gel in 1× TBE (Tris-borate-EDTA) containing 0.5 μg of ethidium bromide per ml. The combined results of the core and 5'NCR RFLP analyses were used to predict the genotype of HCV within the samples.

A)

a1	94	101	38	24	148	
a2	94	101	62		148	
a3	94	139		24	148	
a4	94	139		72		
a5	94	139		24	64	84
a6	233			24	148	
a7	94	52	87	24	148	
a8	94	52	87	172		
a9	94	101	38	172		
a10	94	163		148		
a11	94	51	260			
a12	94	311				
a13	94	101	210			

B)

s1	94	163	148	
s2	257		148	
s3	94	52	259	
s4	94	52	111	148
s5	94	311		

FIG. 1. Restriction endonuclease cleavage patterns of amplified core sequences using enzymes *Ava*I (A) and *Sma*I (B) for published variants and samples analyzed in the current study. The numbers are the sizes (in base pairs) of the DNA fragments produced.

Direct sequencing of PCR products. Samples showing restriction patterns different from those predicted from published sequence data were reamplified by using biotinylated primers. In order to obtain single-stranded DNA for sequencing, the biotinylated products were bound onto paramagnetic streptavidin-coated beads (Dynabeads M280; Dynal) as previously described (12). Dideoxy-termination sequencing reactions were performed by using the Sequenase sequencing kit from United States Biochemical Corp. according to the manufacturer's instructions, except that reactions were carried out in 10% dimethyl sulfoxide and the template DNA was heat denatured before primer annealing. Sequences were read manually from autoradiograms and analyzed by using standard sequence software.

Phylogenetic analysis. Phylogenetic analysis was carried out by using the program NEIGHBOR in the PHYLIP package, as previously described (20).

Nucleotide sequence accession numbers. New sequences obtained in this study have been submitted to GenBank and have been assigned accession numbers L49473 to L49485, respectively.

RESULTS AND DISCUSSION

Development of core RFLP assay. Published nucleotide sequences in the core region of types 1 and 6 and novel variants that grouped with type 6 upon phylogenetic analysis were analyzed with DNAnalyz software to identify restriction enzymes that recognized sequence polymorphism between HCV genotypes. Cleavage with *Ava*I and *Sma*I consistently showed distinct predicted restriction patterns for type 1 and non-type 1 sequences (Fig. 1; Table 1). From the total of 13 different restriction patterns obtained using *Ava*I (a1 to a13 [Fig. 1A]) and the five patterns obtained using *Sma*I (s1 to s5 [Fig. 1B]), published sequences of the type 6 group produced a total of three combinations of patterns.

TABLE 1. Association of different HCV genotypes with RFLP patterns in the core region

Genotype	No. of samples with restriction pattern[a]:													Total no. of samples
	a1s1	a2s1	a3s1	a4s1	a4s5	a5s1	a6s5	a7s4	a8s3	a9s2	a10s1	a11s5	a12s5	a13s5	
1a	14	2													16
1b			43		5										48
6a													30		30
T6G[b]											2		15	1	18
Total no. of samples	14	2	43		5						2		45	1	**112**

[a] Combination of restriction patterns as defined in Fig. 1; a1s1 corresponds to pattern 1 with *Ava*I and pattern 1 with *Sma*I.
[b] Variants of HCV within the type 6 clade but excluding the previously classified type 6a genotype.

Among published sequences, the most common restriction pattern for type 1a sequences was a1s1 (n = 14), while the majority of type 1b sequences showed the a3s1 pattern (n = 43) (Table 1). With the exception of three sequences (VN540, VN787, and VN507), all variants in the type 6 group (including type 6a) could be predicted to produce the a12s5 pattern upon cleavage with *Ava*I and *Sma*I (Table 1). In every case, RFLP patterns distinct from those of type 1 variants were obtained.

Geographical distribution of surveyed samples. To investigate the distribution of type 6 group variants in SE Asia, a total of 108 samples from Malaysia, Thailand, Macau, Hong Kong, Singapore, Burma, and Taiwan that had been previously identified as type 1 (9) were retested in the new core RFLP assay (Table 2). A much wider range of restriction patterns than was obtained from our analysis of published sequences was obtained from this survey. Although the vast majority of samples produced RFLP pattern a1s1 or a3s1 and could therefore be identified as type 1a or 1b, the remaining 26 samples produced a total of 11 distinct RFLP patterns with the two enzymes, many of which corresponded to those previously associated with type 6 group variants (Table 1).

Each of the 26 samples was sequenced in the core region to make a definitive identification of the genotype. Of the 26 samples, 18 could be classified as type 1, while the remaining 8 contained sequences in the core region that placed them within the type 6 group (see below). These type 6 group sequences were found in samples from Thailand (n = 7) or Burma (n = 1) and showed the restriction pattern a11s5 or a12s5 (Table 2).

Phylogenetic analysis of novel genotypes. To demonstrate the relationship between the eight type 6 group variants with those previously found in Vietnam and Thailand, we carried out phylogenetic analysis of sequences from the core and NS-5 regions of these variants (Fig. 2 and 3). All eight core sequences grouped with the type 6 clade and showed various relationships with variants previously found in Vietnam and Thailand (13, 15) (Fig. 2). Three samples from Thailand (EUTH5230, EUTH7, and EUTH13) grouped closely with variants previously found in Thailand and provisionally classified as NGII (15). The sample from Burma was closest to but distinct from variants in Thailand referred to as NGI, while the remaining four samples (EUTH1, EUTH21, EUTH22, and EUTH39) showed a distant relationship to variants from Vietnam described as type 9a (13).

To investigate further the relationship of the new variants to those previously found in SE Asia, we compared sequences in the more variable NS-5 region (Fig. 3). Each new sequence clustered within the type 6 group, although the addition of the new sequences from Thailand increased still further the diversity within the group as a whole. Sequence relationships found in the core region among members of the type 6 group were closely reproduced in the NS-5 region. For example, the NS-5 sequences of EUTH1 and EUTH22 were distinct from all previously analyzed variants in the type 6 group but were closest to VN004 and VN085 from Vietnam, which were described as type 9a (13). Similarly, the close relationship between EUTH5230, EUTH7, and EUTH13 with NGII in the core region was also found in NS-5. The NS-5 sequence of the sample from Burma (EUBUR1) was found to be the same genotype as variants B4-92 and PC found in northern Thailand (1).

Identification of type 6 group sequences in the 5′NCR. All published type 6a sequences in the 5′NCR are distinct from type 1 sequences, because they contain characteristic insertions (CA at position −146 and C at position −138) which are generally not present in other genotypes (22). However, other variants in the type 6 group show a range of different 5′NCR sequences, none of which contain the CA insertion at position −146 and which infrequently show the C insertion at position

TABLE 2. Distribution of different core RFLP patterns in the places surveyed

Place	No. of samples with restriction pattern[a]:														Total no. of samples
	a1s1	a2s1	a3s1	a4s1	a4s5	a5s1	a6s5	a7s4	a8s3	a9s2	a10s1	a11s5	a12s5	a13s5	
Burma			2										1		3
Thailand	9		9	1	1		1					4	3		28
Malaysia	9	1	5					2	1						18
Singapore			1		1										2
Hong Kong	3		38	1			1								43
Macau	1		2	1		1				1	2				8
Taiwan			3			1					2				6
Total no. of samples	22	1	60	3	2	2	2	2	1	1	4	4	4	0	**108**

[a] Combination of restriction patterns as defined in Fig. 1; a1s1 corresponds to pattern 1 with *Ava*I and pattern 1 with *Sma*I.

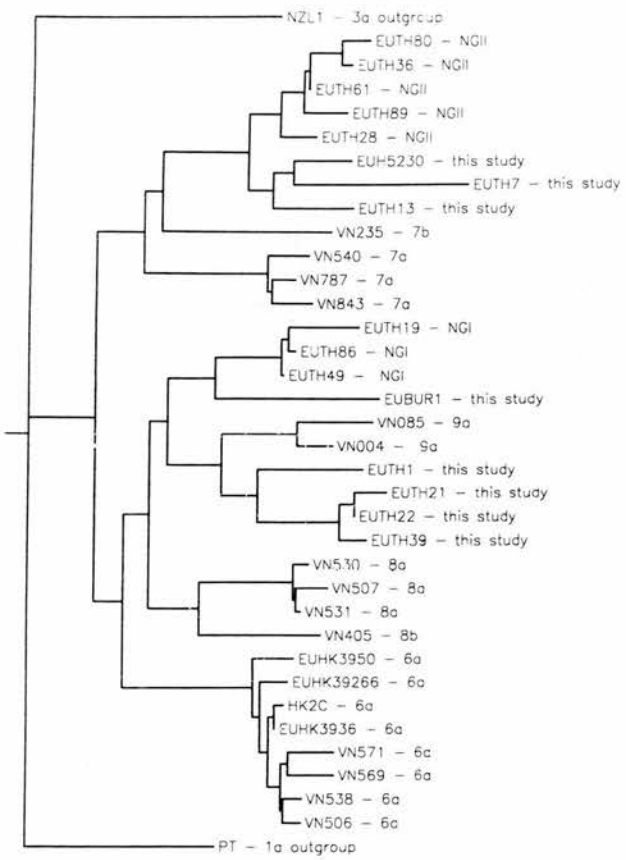

FIG. 2. Phylogenetic analysis of core sequences from the type 6 clade including those of eight variants identified in the current group. The phylogenetic tree was rooted by using sequences from HCV-PT (type 1a [8]) and NZLI (type 3a [18]) as outgroups. Previously published provisional genotype assignments are indicated.

−138. Furthermore, few nucleotide differences are found between these variants and type 1. In the extreme case, for VN540, VN843, VN235, VN507, VN530, and VN531, the 5′NCR sequences were identical to those of type 1b and the 5′NCR sequence of VN085 was identical to that of type 1a (Fig. 4). On the other hand, most of the variants from Thailand showed 5′NCR sequences that differed at several sites from type 1 sequences; in the case of sequences previously described as NGI (15), a conserved pair of covariant changes in the proposed stem-loop region 3 (3) that could be identified by cleavage with *Hpa*II (which recognizes the CCGG sequences present in type 1 sequences but which is disrupted by the substitution of A for C at position −155 in NGI sequences) was found. Cleavage of 5′NCR sequences using the combination of *Dde*I and *Hpa*II from NGI produced pattern B (Fig. 5), clearly distinguishable from pattern A found in all type 1 5′NCR sequences analyzed to date. A different covariant change in the same stem-loop structure was found among the sequences described as NGII. In this case, cleavage with *Dde*I identified the polymorphism at position −127 (G for A in NGII) and produced pattern C with the *Dde*I-*Hpa*II enzyme combination (Fig. 5). Although pattern D (produced by type 6a 5′NCR sequences [22]) is different from pattern A for type 1, the 3-base size difference of the large fragment (127 and 130 bp) would not necessarily be identifiable by agarose gel electrophoresis of the cleaved DNA (9).

With this limited ability to identify at least a proportion of type 6 group variants, we used the *Dde*I-*Hpa*II enzyme combination to cleave amplified 5′NCR sequences from the survey population. All 100 sequences that were classified as type 1 by RFLP or sequence analysis of the core region produced pattern A. Among the 8 samples identified in this study as belonging to the type 6 group, EUBURI produced pattern B, while EUTH5230, EUTH7, and EUTH13 produced pattern C. EUTH1, EUTH21, EUTH22, and EUTH39, which were not closely related to any of the variants within the type 6 group, produced pattern A (and had sequences in the 5′NCR similar or identical to type 1b [data not shown]). From this analysis it can be seen that only half of the type 6 group variants would have been identified even when using new enzymes designed to detect observed sequence differences between NGI and NGII with type 1 sequences.

HCV genotype distribution in SE Asia. The identification of only eight samples in the type 6 group of HCV genotypes indicates an extremely limited distribution of these variants in most of the places surveyed. Seven of these variants were found in Thailand, consistent with previous observations that they represent one of the principal variants (with type 3) in blood donor and hepatitis C patient populations (1, 15). Type 6 group variants (including type 6a) are well documented in Vietnam (26), and the finding of a single example of this genotype in Burma hints at a wider westward distribution in SE Asia.

In attempts to review current information from other groups

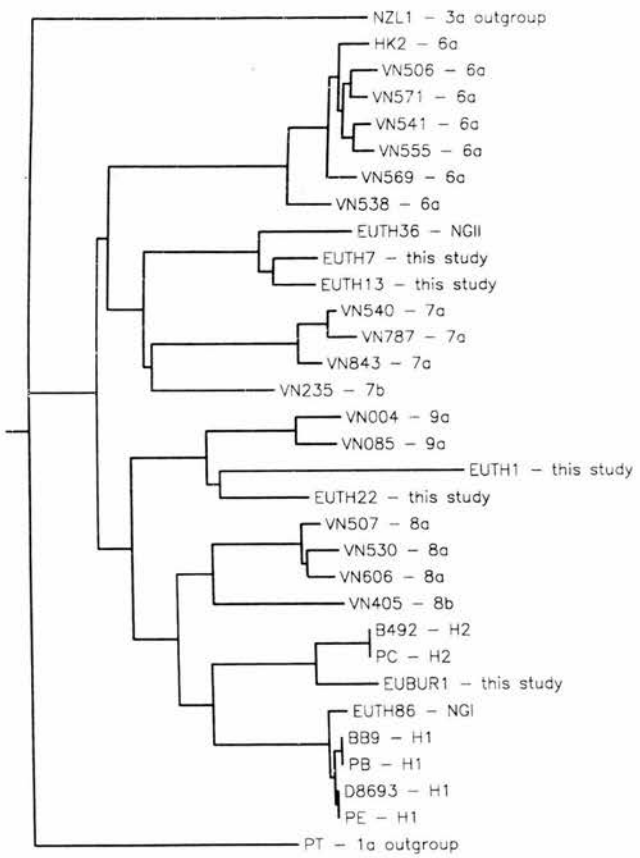

FIG. 3. Phylogenetic analysis of NS-5 sequences from the type 6 clade including those of eight variants identified in the current group. The phylogenetic tree was rooted by using sequences from HCV-PT (type 1a [8]) and NZLI (type 3a [18]) as outgroups. Previously published provisional genotype assignments are indicated.

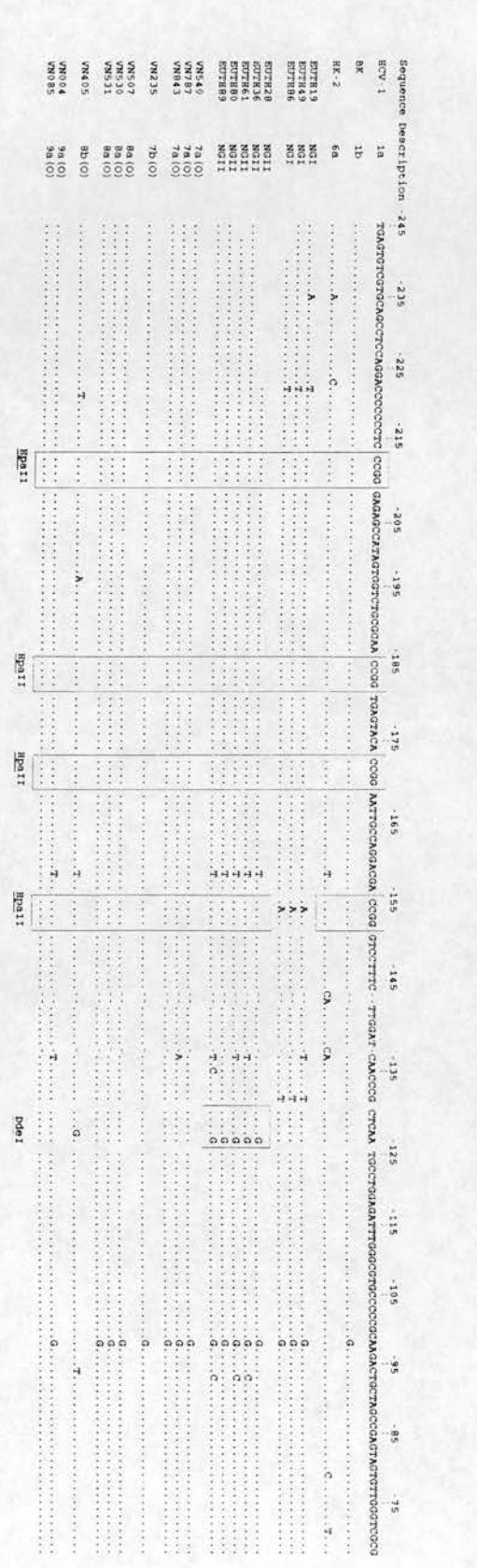

FIG. 4. Comparisons of sequences in the amplified portion of 5′NCR from type 6 group variants. Nucleotides different from those of HCV-PT are indicated; nucleotides are numbered as in reference 8. Recognition sequences for the restriction endonucleases *Hpa*II and *Dde*I are in boxes. Sequences are labelled type 7a, 7b, 8a, 8b, 9a, NGI, and NGII (Description column) according to their original descriptions (15, 26).

A	67	27	12	18	127	
B	67	27	12	145		
C	67	27	12	18	25	102
D	67	27	12	18	130	

FIG. 5. DNA fragments produced upon cleavage of amplified 5′NCR sequences from type 1 and type 6 groups using restriction endonucleases *Dde*I and *Hpa*II. The numbers are the sizes (in base pairs) of the DNA fragments produced.

on the distribution of HCV genotypes in SE Asia, most data obtained from genotyping assays such as the RFLP assays (9), InnoLipa (23), and assays using type-specific primers in the core region (17) cannot be relied upon because of the limited range of HCV variants that can be identified. In the current study, we have shown that type 6 group variants may appear as type 1 in an RFLP assay based upon the 5′NCR, whether or not it is modified by the use of additional restriction enzymes such as *Dde*I and *Hpa*I. In this respect, the survey recently published by Greene et al. (10) is particularly informative, because genotype assignments were made by nucleotide sequence determinations of several regions of the HCV genome. The absence of type 6 group variants from a total of 45 samples collected from Singapore, Indonesia, Philippines, and South Korea is consistent with the finding in this study of a restricted distribution of type 6 group variants in Malaysia, Singapore, Taiwan, Macau, and Hong Kong. However, the survey by Greene et al. (10) failed to detect type 6 group variants in Thailand (3 type 1a, 8 type 1b, and 10 type 3a and 3b variants) and appears inconsistent with their frequent detection in this study and previous studies (1, 15). It is possible that the frequency of infection with type 6 group variants differs between geographical regions within Thailand, or it is possible that there are differences in its distribution in different risk groups for infection or age of subjects.

Figure 6 attempts to summarize current knowledge of genotype distributions of HCV throughout SE Asia and the Far East using information obtained from this study and previously published information (9, 10, 26). The pie charts represent the relative frequencies of genotypes 1 to 6 as determined by sequence comparisons in coding regions (9, 15, 26), RFLP analysis in the 5′NCR (9), and genotyping using type-specific primers in the core region (26). Figure 6 includes a separate representation of type 6 group variants (indicated as NG, distinct from type 6a) in the three countries where it has been detected so far (Vietnam, Thailand, and Burma).

Although the number of samples from some places was small, this combined analysis reveals some clear geographical trends in relative genotype frequencies. Type 1b variants appear ubiquitous throughout the region, and a steady increase in the frequency of type 3 infection in more westward countries is apparent, which is not found in the Far East. Type 6 group variants were found in a single, possibly continuous block that includes Vietnam, Thailand, and Burma but were not detected elsewhere. The distribution of type 6 group variants overlaps in Vietnam with type 6a, with the latter also being prevalent in Hong Kong and Macau.

Little is currently understood concerning the past epidemiology of HCV transmission that is responsible for the current distribution of HCV variants. However, the information obtained in this study will be of future use in the understanding of HCV sequence variation and the ancestry of the currently identified HCV genotypes. In particular, the overlapping ranges of type 6a with other variants in the type 6 clade in the mainland of SE Asia provides an insight into their interrela-

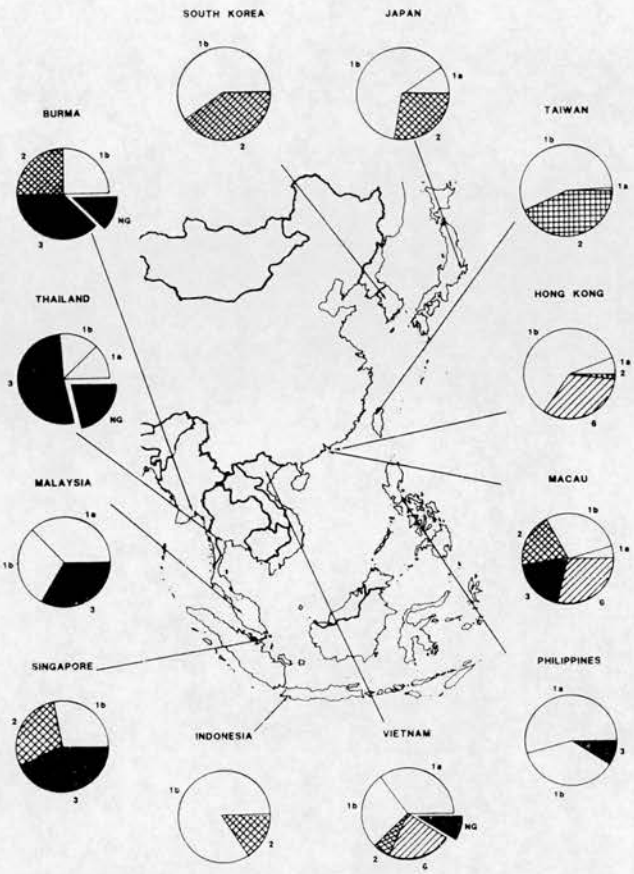

FIG. 6. Map of SE Asia summarizing the HCV genotype distributions identified in the current study and by previously published analyses. Pie charts indicate the proportions of various HCV genotypes in each place surveyed, although the sample numbers available were small in some places, such as Burma. In addition to the current study, data for Indonesia, Philippines, and South Korea were obtained from reference 10, data for Vietnam were from reference 26, and data for Japan were from sequence information in GenBank.

tionship that will be of value in future evolutionary analyses of these sequences.

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Distribution of Hepatitis C Virus Genotypes Determined by Line Probe Assay in Patients with Chronic Hepatitis C Seen at Tertiary Referral Centers in the United States

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Objective: To 1) verify the validity of a new line probe assay for hepatitis C virus (HCV) genotyping and 2) determine the distribution of HCV genotypes and the association between HCV genotype and clinical variables in patients with chronic hepatitis C seen in tertiary referral centers in the United States.

Design: Retrospective cross-sectional analysis.

Patients: 438 patients with chronic hepatitis C from 10 tertiary referral centers.

Measurements: The validity of the line probe assay was first verified against a panel of serum specimens that had previously been characterized by six different HCV genotyping methods. Specimens from all 438 patients were then genotyped using this line probe assay. The associations between HCV genotype and clinical variables were examined using analysis of variance. Pairwise testing was used when the *F* test showed a statistically significant difference. Nonparametric alternatives were used for variables for which normality could not be assumed.

Results: The line probe assay was quick and reproducible, and it showed good concordance with other tests. In our sample, the proportions of patients with HCV types 1, 2, 3, and 4 were 71.5%, 13.5%, 5.5%, and 1.1%, respectively. Subtypes 1a and 1b were seen in approximately equal proportions of patients with HCV type 1. Mixed infection was detected in 3.7% of specimens, and 4.8% of specimens either had negative results on polymerase chain reaction or could not be typed. A higher proportion of patients with HCV type 1 than of patients with HCV–type 1 had acquired HCV through transfusion of blood products (50% compared with 25%; $P < 0.001$). Patients with HCV type 1 also had a longer estimated duration of infection compared with patients with HCV type 3 ($P = 0.004$) and type 4 ($P = 0.049$). Disease activity did not differ among patients infected with HCV types 1, 2, or 3. Levels of viremia were similar in patients with HCV types 1, 2, or 3, but patients with HCV type 4 had a lower level of viremia than did patients with HCV type 1 ($P = 0.047$).

Conclusions: The line probe assay can be used in patients with chronic HCV infection in the United States. In patients with chronic hepatitis C referred to tertiary centers in the United States, type 1 is the most common HCV genotype. Disease activity and viremia levels do not differ among patients chronically infected with HCV types 1, 2, or 3.

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Hepatitis C virus (HCV) has a high spontaneous mutation rate with an estimated frequency of 1.4 to 1.9×10^{-3} mutations per nucleotide per year (1–3). As a result, HCV exists as a heterogeneous group of viruses sharing approximately 70% homology. On the basis of nucleotide sequence homology, HCV has been classified into six major genotypes and a series of subtypes. A proposed consensus system for nomenclature, based on sequence homology in at least two regions with confirmation by phylogenetic tree analysis, has been adopted by most HCV investigators (4). The relation between this nomenclature and other commonly used nomenclature systems is shown in Table 1.

Various methods have been used for HCV genotyping, including genomic amplification and sequencing (10, 14–16), polymerase chain reaction (PCR) with genotype-specific primers (5, 8), restriction fragment length polymorphism of the PCR amplicons (13, 17), differential hybridization (18), and serologic genotyping (11, 19). Genomic amplification and sequencing, followed by sequence comparison and phylogenetic tree construction for confirmation, is currently considered the gold standard; the genomic regions commonly used for this approach include the HCV core region, envelope 1, and nonstructural region 5 (NS5). This method, however, is expensive and labor intensive, making it difficult to study HCV genotypes in a large number of patients.

Several alternative methods have been proposed. Okamoto and colleagues (5) and Chayama and coworkers (8) have described PCR done with genotype-specific primers derived from the HCV core region and from NS5, respectively. These methods rely on the use of genotype-specific primers that anneal to sequences unique to specific HCV genotypes. Genotype-specific primers derived from the HCV core region allowed the differentiation of HCV types 1a, 1b, 2a, and 2b (5); genotype-specific primers derived from NS5 allowed the differentiation of HCV types 1a, 1b, 2a, 2b, and 3b. Restriction fragment length polymorphism of the PCR amplicons relies on the presence of unique and genotype-specific nucleotide substitutions that are recognized and digested by restriction enzymes into fragments that can be separated be gel electrophoresis (13, 17).

The 5′ untranslated region (5′UTR) is commonly used for this approach. Hybridization of PCR amplicon mounted on a solid phase using genotype-specific probes has also been used for HCV genotyping (18). Finally, serologic approaches have also been used to determine HCV genotypes. These approaches rely on the different amino acid sequences encoded by different nucleotide sequences from different HCV genotypes. Patients infected with different HCV genotypes may therefore have antibodies directed to genotype-specific amino acid sequences. Polypeptides and synthetic peptides derived from nonstructural region 4 (NS4), as well as synthetic peptides derived from the HCV core region, have been used for serologic genotyping (11, 19). These peptides are used to coat enzyme immunoassay plates, and specific binding by a serum specimen is determined by peptide competition. The HCV genotypes assigned by this method are commonly referred to as serotypes. Conventionally, the term "HCV serotype" refers to different viral types determined by a panel of cross-neutralization antibodies. Because recognition of genotype-specific peptides by a patient's antibody response reflects the difference in amino acid sequences and thus nucleotide sequences, the results of this approach are more appropriately referred to as "serologically defined genotypes" or "serologic genotypes."

The concordance among these different HCV genotyping methods has been questioned. In a previous study (20), we compared six different genotyping methods and found that most of them had high concordance with each other. The only method that we identified as unsuitable for U.S. patients was PCR with genotype-specific primers derived from the HCV core region, proposed by Okamoto and colleagues (11). This method gave false-positive signals for HCV type 1b in patients who were actually infected with types 1a and 3a (20).

Because all molecular biological genotyping methods rely on PCR as a first step, those systems based on the 5′UTR, the most conserved genomic region, should be the most sensitive. Recently, a new genotyping system based on reverse hybridization of the labeled PCR amplicon derived from the 5′UTR (line probe assay, INNO-LiPA HCV, Innogenetics, Ghent, Belgium) was developed and has been used widely by investigators in Europe (21, 22). Whether this line probe assay is also useful in patients with chronic HCV infection in the United States is unknown.

In this study, we sought to 1) assess the concordance of this line probe assay with other assays on a panel of well-characterized serum specimens to verify the line probe assay's validity; 2) determine the distribution of various HCV genotypes in a large sample of patients with chronic hepatitis C seen in tertiary referral centers in the United States; and 3) evaluate the clinical characteristics of patients infected with different HCV genotypes.

Table 1. Commonly Used Hepatitis C Virus Genotype Nomenclature Systems*

Okamoto/ Mori (5–7)	Chayama (8)	Houghton/ Cha (9, 10)	Kohara (11)	Enomoto (12)	Simmonds (13)	Proposed System (4)
I	I	I	I	PT	1a	1a
II	II	II	I	K1	1b	1b
						1c
III	III	III	II	K2a	2a	2a
IV	IV	III	II	K2b	2b	2b
		III				2c
V		IV			3	3a
VI	V	IV				3b
						4a
		V				5a
						6a

Methods

Patients

We studied 438 patients from three different groups (Table 2). All patients were from the United States, had chronic hepatitis C, and were seen in tertiary referral centers. Most had been referred by their physicians or by a gastroenterologist or hepatologist for inclusion in experimental antiviral therapy programs. The first group consisted of 137 patients with chronic HCV infection whose serum specimens had previously been characterized with six different genotyping methods (20). The specimens of these patients were studied to verify the validity of the line probe assay. The second group consisted of 248 patients from nine centers in the United States who had participated in a randomized, controlled study of interferon-α therapy. One of these 248 patients was negative for the antibody to HCV (anti-HCV), and another had no serum specimen available, which left a total of 246 patients for this study. A careful review of the serum bank showed that 40 patients were included in both group 1 and group 2. To avoid duplication, we used only the specimen obtained from each patient before interferon-α therapy was started. For patients for whom serum specimens obtained before treatment on the day of study entry were not available, specimens collected 1 or 2 months before treatment were used for HCV RNA quantitation and genotyping. A previous study (23) has shown that the viremia levels of patients with chronic HCV infection usually remain about the same over time. The details of the role of HCV genotype as a predictor of subsequent response to interferon-α therapy will be discussed in another report (Lindsay KL and

Table 2. Clinical, Biochemical, and Histologic Characteristics of Study Patients

Characteristic	Group 1 (n = 97)	Group 2 (n = 246)	Group 3 (n = 95)
Male:female ratio	69:28	175:71	45:50
Mean age (range), y	47 (19–73)	46 (18–74)	45 (18–76)
Mode of acquisition of HCV, n†			
Blood product transfusion	11	111	15
Health care workers	0	8	1
Intravenous drug use	3	87	17
Sporadic	2	40	21
Sexual	0	0	1
Not documented	81	0	40
Median estimated duration of HCV infection (range), y ‡	10.0 (1–26)	8.2 (1–41)	4 (1–26)
Mean serum alanine aminotransferase level ± SD, U/L	136 ± 113	189 ± 109	123 ± 117
Positive for anti-HCV§, n	97	246	95
Positive for serum HCV RNA, n‖	97	208	91
Median serum HCV RNA level (range), × 10^6 *genome eq/mL*¶	4.1 (PCR^+ to 46.7)	2.61 (PCR^- to 75.2)	3.6 (PCR^- to 105.1)
Histologic characteristics**			
Median Knodell score (range)	10 (3–14)	13 (4–18)	10 (3–14)
Median histologic activity index (range)	6 (2–10)	9 (2–14)	7 (2–10)

* anti-HCV = antibody to hepatitis C virus; HCV = hepatitis C virus; PCR^+ = positive results on polymerase chain reaction; PCR^- = negative results on polymerase chain reaction.
† Available in 317 patients.
‡ Available in 258 patients.
§ By enzyme immunoassay.
‖ By polymerase chain reaction.
¶ By polymerase chain reaction and branched-DNA assay.
** Available in 262 patients.

colleagues. In preparation). The third group consisted of 95 patients seen at the University of Florida in a prospective study of immune-mediated mechanisms of hepatocellular damage.

All patients studied were seropositive for anti-HCV and had had abnormal serum aminotransferase levels for at least 6 months. None had biochemical or serologic evidence of other causes of liver disease; in particular, all were seronegative for hepatitis B surface antigen. Duration of HCV infection was established by detailed history taking and was estimated by the clinical investigator (available in 258 patients).

Antibody to Hepatitis C Virus and Detection and Quantitation of Hepatitis C Virus RNA

We detected anti-HCV by using second-generation enzyme immunoassay (Ortho Diagnostics, Raritan, New Jersey or Abbott Diagnostics, North Chicago, Illinois). Serum specimens were tested for HCV RNA by reverse transcription "nested" PCR with primers derived from the highly conserved 5′UTR and quantitated by branched DNA (bDNA) signal amplification assay (Quantiplex HCV RNA, version 1.0, Chiron Corp., Emeryville, California) as described previously (24, 25). All reverse transcription PCR assays were done in a single laboratory; the performance of the PCR assays has been previously verified to have specificity and sensitivity of 100% against a coded serum panel prepared by the Chiron Corporation.

Serum HCV RNA levels were measured by bDNA signal amplification assay (bDNA, Quantiplex HCV, version 1.0, Chiron Corp.). Because the bDNA assay underestimates HCV RNA levels in patients infected with HCV types 2 and 3, the appropriate correction factors (×3 for type 2 and ×2 for type 3) were applied to obtain accurate levels of viremia (26–28). The bDNA assay accurately measures HCV RNA levels for HCV types 1, 4, 5, and 6; thus, no conversion was necessary for these types.

Hepatitis C Virus Genotyping

The details of the six HCV genotyping methods used to characterize the serum specimens of group 1 have been described previously (20). These methods are PCR with genotype-specific primers based on the HCV core region and the genomic region of NS5, restriction fragment length polymorphism based on the 5′UTR, direct sequencing of the NS5B region, and serologic genotyping based on NS4 recombinant and synthetic peptides.

The line probe assay was used to assess HCV genotyping as previously described (21). Briefly, the 5′UTR was amplified using "nested" PCR with biotinylated primers. The labeled amplicon was allowed to hybridize with probes derived from various HCV genotypes mounted on a strip. After stringent washing, streptavidin labeled with alkaline phosphatase was used to trace the hybridized products, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate were used as substrates. To ensure that this assay was consistently concordant with other tests, 342 specimens were tested by restriction fragment length polymorphism of the PCR amplicon generated from the 5′UTR, and 339 specimens were tested by a serologic genotyping assay based on the use of peptides derived from two regions of NS4 (amino acid positions 1691 to 1708 and 1710 to 1728) as capture antigens (20, 29).

The HCV genotype nomenclature used in this report is that proposed by an international panel (4) and is the most widely accepted of all nomenclature systems. We use "genotype" as a general term. The major types, designated 1, 2, 3, and so on are referred to as "types," and the subtypes a, b, c, and so on are referred to as "subtypes" (Table 1).

Hepatitis C virus types 2a and 2c cannot be distinguished on the basis of the 5′UTR, but they clearly separate into different subtypes by phylogenetic tree analysis of the subgenomic region of NS5 (30). Therefore, it is possible that specimens genotyped as 2a were in fact 2c. In our study, the NS5B nucleotide sequences from three patients with HCV type 2a were determined by the dideoxy chain termination method and analyzed with both neighbor-joining and maximum likelihood analyses. All three were specimens assigned as HCV type 2a (data not shown).

Liver Histology

Baseline liver biopsy specimens were available for 262 patients, and the investigators assessing these specimens were blinded. The Knodell score and its individual components (periportal, lobular, and portal inflammation and fibrosis) (31) and the histologic inflammatory index (summation of the lobular, periportal, and portal inflammation scores) (32) were assessed.

Statistical Analysis

All data were analyzed using SPSS for Windows (SPSS, Inc., North Chicago, Illinois). Once a specimen was included in the study, the data were used for the final analysis; this includes serum specimens that were negative on reverse transcriptase PCR. This approach is similar to the intention-to-treat analysis in clinical studies on therapeutic agents.

For the analysis of clinical associations, we used analysis of variance, which assumes normality, to compare the means for the variables across groups. For those cases in which the F test indicated statistically significant differences, pairwise multiple comparisons using the Student–Neuman–Keuls test were applied. The Mann–Whitney rank-sum test and the Spearman rank correlation coefficient were applied for those variables for which normality could not be assumed.

Results

Line Probe Assay

Amplification of the 5′UTR using the labeled primers and subsequent genotyping by the line probe assay have a sensitivity similar to that of our in-house PCR assay, which is also based on the 5′UTR (our in-house assay was determined to have a consistent sensitivity <1000 genomes/mL; in most runs, our detection limit was <500 genomes/mL). The line probe assay was repeated on 25 specimens, and the results were 100% reproducible on this small number of specimens. The line probe assay, which relies on differential hybridization of PCR amplicons derived from the 5′UTR, is probably more sensitive in identifying mixed infection than other genotyping methods, which amplify less conserved regions of the genome. Accordingly, if a specimen is found to have genotypes 1a and 3a by the line probe assay and 1a by another method, the results should still be considered concordant.

In the 137 specimens that had previously been genotyped by six other methods, the concordance of the line probe assay and the other methods was 100% (specificity of 100%). In the 342 specimens that were genotyped by both restriction fragment length polymorphism analysis of the 5′UTR and line probe assay, the concordance of the two methods was 98.2%. The results were discordant in 6 specimens (in 1 specimen, restriction fragment length polymorphism assigned type 1 and the line probe assay assigned type 2; in 5 specimens, restriction fragment length polymorphism assigned type 2 and the line probe assay assigned type 1). Of the 339 specimens that were serologic genotyped on the basis of the NS4 genomic region, the line probe assay and serotyping had a concordance of 98.5%. Five specimens were discordant (in 1 specimen, serologic genotyping assigned type 1 and the line probe assay assigned type 2; in 1 specimen, serologic genotyping assigned type 1 and the line probe assay assigned type 3; and in 3 specimens, serologic genotyping assigned type 2 and the line probe assay assigned type 1).

Three patients who were positive by PCR as determined by ethidium bromide staining showed no hybridization signals on the line probe assay strips. Overall, the line probe assay was able to assign HCV genotype in 417 of the 421 specimens (99.1%) that were positive for HCV RNA by PCR. If all 438 specimens tested were considered, the line probe assay assigned HCV genotype to 95.2%.

Distribution of Hepatitis C Virus Genotypes

On the basis of the line probe assay, the distribution of various HCV genotypes in patients with chronic hepatitis C seen in tertiary referral centers in various regions of the United States was evaluated (Table 3). Several important points emerged. First, 96.1% of the specimens were positive by PCR, indicating that these specimens were fairly well preserved. Second, types 1a and 1b were the most common HCV genotypes in this population. Third,

the overall distribution of various HCV types in different geographic locations was similar.

Clinical Characteristics of Patients Infected with Different Hepatitis C Virus Genotypes

For patients infected with HCV type 1, genotyping by the line probe assay may show the following possible patterns: type 1a; type 1b; type 1a plus type 1b; and type 1 in which subtype a or b could not be defined. As a first step, we compared all of the subtypes within the major types, and no differences were seen in clinical characteristics between the subtypes within the major types. In particular, no differences in any clinical variables were seen between patients infected with HCV type 1a and those infected with HCV type 1b. Therefore, patients with each major HCV type were grouped together regardless of subtype (for example, patients with type 1a, type 1b, type 1a plus type 1b, and type 1 without specified subtype were all grouped together as HCV type 1). Patients with mixed infection were combined into a single group, and patients who had negative results on PCR were also considered as a group.

No difference in age was seen among patients infected with HCV types 1, 2, and 4. Patients infected with HCV type 3 were younger than patients infected with type 1 and type 2 (type 3 compared with type 1 [$P = 0.002$]; type 3 compared with type 2 [$P = 0.004$]; *see* Table 4). No difference in sex distribution was seen among patients infected with different HCV genotypes. Of the 258 patients in which the estimated duration of HCV infection was documented, no difference in duration was seen between patients infected with HCV type 1 and those infected with HCV type 2. However, patients infected with HCV type 1 had a longer estimated duration of infection than did patients infected with types 3 and 4 ($P = 0.004$ for type 3 and $P = 0.049$ for type 4). Of the 317 patients in whom the method of acquisition was documented, 108 of 216 (50%) of the patients with HCV type 1 infection had acquired HCV through the transfusion of blood products compared with 16 of 65 (25%) of the patients infected with another HCV type ($P < 0.001$; Table 4).

In this data set, no statistical differences were seen in serum alanine aminotransferase or aspartate aminotransferase levels among patients infected with different HCV genotypes, except that the few patients infected with HCV type 4 had lower serum alanine aminotransferase levels (70 ± 38 U/L [$n = 5$]) than patients infected with other HCV types (ranging from 154 ± 118 U/L [$n = 24$] for HCV type 3 to 163 ± 118 U/L [$n = 313$] for HCV type 1), but this did not reach statistical significance ($P = 0.07$; Table 4).

Of the 262 patients who had baseline liver biopsy specimens, 182 had HCV type 1, 35 had type 2, 12 had type 3, and 2 had type 4. Because so few patients with HCV type 4 infection had liver biopsy specimens available, analysis was done only on patients with HCV types 1, 2, and 3 (Table 4). No significant difference was seen in the total histologic inflammatory index or Knodell scores among patients infected with different HCV genotypes. Additionally, no differences were seen in individual histologic scores (lobular, periportal, and portal inflammation and fibrosis) among patients infected with different HCV types. The only exception was that patients with HCV type 2 infection had more periportal inflammation (mean rank, 124.4; $n = 35$) than did patients with type 1 infection (mean rank, 106.0; $n = 182$),

Table 3. Prevalence of Various Hepatitis C Virus Genotypes in Different Areas of the United States Based on the Line Probe Assay*

Location	Total Patients	Hepatitis C Virus Genotype												
		1	1a	1b	1a+1b	2	2a	2b	2a+2b	3a	4	Mixed	Undetermined but PCR$^+$	PCR$^-$
	←						*n*†							→
Gainesville, Florida	142	97	56	37	4	29	8	19	0	9	5	2	0	0
St. Louis, Missouri	61	49	22	23	0	5	2	2	0	3	0	1	0	3
Los Angeles, California	59	46	21	24	0	7	2	2	3	2	0	4	0	0
Miami, Florida	34	21	8	8	3	3	0	0	3	0	0	2	0	8
New Orleans, Louisiana	30	24	11	12	1	1	1	0	0	1	0	1	2	1
Providence, Rhode Island	30	17	8	6	1	4	1	2	1	4	0	4	1	0
Boston, Massachusetts	28	22	14	5	2	1	0	1	0	2	0	0	1	2
Denver, Colorado	24	17	9	8	0	4	1	0	2	1	0	0	0	2
Louisville, Kentucky	14	6	3	2	0	4	1	1	1	1	0	2	0	1
San Francisco, California	16	14	8	6	0	1	0	1	0	1	0	0	0	0
Total patients, *n* (%)	438	313 (71.5)	160 (36.5)	131 (29.9)	11 (2.5)	59 (13.5)	16 (3.7)	28 (6.6)	10 (2.3)	24 (5.5)	5 (1.1)	16 (3.7)	4 (0.9)	17 (3.9)

* The difference between patients with type 1 and total patients with types 1a, 1b, and 1a+1b represents the number of patients whose genotypes were assigned as type 1 but subtype (a or b) was not assigned by line probe assay. The same is true for type 2. PCR$^+$ = positive results on polymerase chain reaction; PCR$^-$ = negative results on polymerase chain reaction.
† *n* refers to number of patients.

Table 4. Clinical, Biochemical, Virologic, and Histologic Characteristics of Patients Infected with Different Hepatitis C Virus Genotypes*

Characteristic	Patients with HCV Type 1 Infection (n = 313)	Patients with HCV Type 2 Infection (n = 59)	Patients with HCV Type 3 Infection (n = 24)	Patients with HCV Type 4 Infection (n = 5)†	Patients with Mixed HCV Infection (n = 16)	Patients PCR or Untypable (n = 21)
Male:female ratio	205:108	34:25	20:4	4:1	13:3	13:8
Mean age ±SD, *y*	47 ± 13	46 ± 11	39 ± 9	44 ± 13	48 ± 13	42 ± 12
Median estimated duration of HCV infection (range)‡, *y*	8.4 (1.0–41.1)	4.6 (1.0–38.2)	2.8 (1.0–10.1)	3.0 (1.0–6.0)	5.3 (1.2–10.6)	11.8 (1.6–34.3)
Mode of acquisition of HCV, *n*§						
Blood product transfusion	108	12	3	1	4	9
Health care workers	2	4	1	0	2	0
Intravenous drug use	73	10	8	4	5	7
Sporadic	33	15	6	0	4	5
Sexual	0	1	0	0	0	0
Median serum HCV RNA level (range), $\times 10^6$ *genome eq/mL*	3.8 (PCR^+ to 105.10)	2.8 (PCR^+ to 99.10)	2.8 (PCR^+ to 41.40)	0.5 (PCR^+ to 32.90)	1.0 (PCR^+ to 88.5)	
Mean alanine aminotransferase level ± SD, U/L	163 ± 118	162 ± 115	154 ± 107	70 ± 38	159 ± 88	188 ± 108
Median Knodell score (range)‖	12 (3–18)	12 (7–15)	12 (6–16)		12 (8–15)	13 (10–17)
Median histologic activity index (range)‖	9 (2–14)	9 (3–12)	9 (4–12)		9 (5–11)	10 (7–14)
Patients with cirrhosis/patients assessed for cirrhosis, *n/n (%)*	71/181 (39)	17/35 (49)	4/12 (33)		5/13 (39)	4/19 (21)

* HCV = hepatitis C virus; PCR = polymerase chain reaction; PCR^+ = positive results on polymerase chain reaction.
† The number in some categories was small (<3), and therefore no data are given, because these patients may not have been adequately representative.
‡ Available in 258 patients.
§ Available in 317 patients.
‖ Available in 262 patients.

but this difference was not statistically significant ($P = 0.088$).

In all patients, HCV viremia levels were documented by reverse transcriptase PCR and quantitated by bDNA assay (Table 4). Patients infected with HCV types 1, 2, and 3 had similar levels of viremia. However, patients infected with HCV type 4 had lower viremia levels than did patients infected with type 1 ($P = 0.047$).

When patients with mixed infection (across types [for example, 1a and 3a] and not within types [for example, 1a and 1b]) were considered as a group, they were older than patients infected with HCV type 3 ($P = 0.018$). No differences were seen in sex distribution, mode of HCV acquisition, or serum biochemical and histologic features between patients with mixed infection and patients infected with other HCV types (Table 4).

The demographic, biochemical, and histologic features of patients who had negative results on PCR did not differ from those of patients in other groups (Table 4).

Discussion

Our study makes several important points. First, for HCV genotyping, the line probe assay based on the 5′UTR is sensitive and reproducible and has good concordance with other tests. Second, the distribution of HCV types 1, 2, and 3 in patients with chronic HCV infection who are referred to tertiary centers in the United States is fairly uniform: Type 1 is the most common HCV type. Third, a higher proportion of patients infected with HCV type 1 than of patients infected with other HCV types appears to have acquired HCV through transfusion of blood products. Fourth, disease activity does not differ significantly among patients infected with HCV types 1, 2, and 3. Finally, patients infected with HCV types 1, 2, and 3 have similar serum HCV RNA levels, whereas patients infected with HCV type 4 have lower levels of viremia than patients infected with type 1.

The line probe assay is relatively simple and standardized. Recently, Smith and associates (30) reported that, theoretically, the line probe assay might assign HCV genotype incorrectly in patients infected with HCV types 3 and 4 and that restriction fragment length polymorphism based on the 5′UTR may be more reliable. Although this may be true in some geographic locations in which more genotypes are present, we show that the HCV genotype assignment produced by line probe assay is highly concordant with that produced by other tests, including restriction fragment length polymorphism, in this field study in the United States. This is probably because HCV types 1 and 2 are the most common HCV genotypes in the United States. In addition, the probes used in the line probe assay have been modified to accommodate the recently identified genetic variations of HCV.

Given that this line probe assay is based on the initial amplification of the 5′UTR, a highly conserved area of the HCV genome, it is not surprising that it is very sensitive. As reported previously (20), genotyping assays based on other genomic regions have lower sensitivity. This is believed to be because of the lower efficiency of amplification related to the higher genetic heterogeneity of these genomic regions. In this previous study of patients in the United States with chronic hepatitis C (20), genotyping based on the HCV core region as described by Okamoto and colleagues has a sensitivity of 89.2%, whereas genotyping based on NS5 has a sensitivity of only 83.5%. The specificity of this line probe assay is also excellent. The concordance of the assay with restriction fragment length polymorphism assay based on the 5′UTR, which we have previously shown to be very reliable (20), was 98.2%.

As of October 1995, the cost of each line probe assay strip was about U.S. $65.00. Adding the cost of PCR, reagents, and supplies and the institutional running cost for PCR and genotyping, excluding technician time, the total cost should be about $100 to $120 (estimated from the cost at the University of Florida). Genotype assignment is reported in a strip form with dark bands. We made photocopies of the strips, and the photocopies seemed to have sufficient quality for evaluation. This will allow investigators to compare results easily. We also sent the photocopies through a standard facsimile machine and found that most of the bands were adequately visible on the facsimile.

On the basis of this line probe assay, HCV type 1 was found to be the most common HCV genotype in patients referred to tertiary referral centers for inclusion in experimental antiviral therapy programs in the United States; it was assigned in 72% of cases. Types 2 and 3 were found in all geographic areas, with average overall distributions of 14% and 6%, respectively. Type 4 was identified in Florida. Recently, type 4 was also found in California (Zhou S and coworkers. In preparation), suggesting that our detection of HCV type 4 only in Florida might be related to the larger number of patients that we studied in that state. Because HCV type 5 is found mainly in South Africa and Belgium, and type 6 is found primarily in Hong Kong and Macau, all of which are developed countries or cities with heavy traffic to the United States, we anticipate that these genotypes will eventually be seen in the United States. We might have identified these types if we had studied more patients.

One potential pitfall of our study was that the persons we studied were referred to tertiary referral centers for consideration for inclusion in experimental antiviral therapy programs in the United States. We attempted to include patients from various geographic locations, and, as discussed above, the distribution of HCV genotypes was fairly similar in all study centers. Our data are important to clinicians and research scientists in the United States, because HCV genotype 1a is only prevalent in the United States, and data generated in other countries will not provide detailed information on the clinical characteristics of patients infected with this HCV type.

Another important issue is whether patients in different phases of infection (for example, healthy blood donors with normal liver test results who are seropositive for anti-HCV, patients with mild liver disease who are seen by primary care physicians, and patients referred for liver transplantation) have the same distribution of HCV genotypes. To date, no published data on the distribution of HCV genotypes in these groups are available. A recent analysis based on more than 100 patients with HCV-related cirrhosis who were referred to the University of California, San Francisco, for liver transplantation showed that these patients had a distribution of HCV genotypes similar to that seen in our study (Zhou S and coworkers. In preparation).

No differences were seen in any of the demographic, clinical, biochemical, virologic, and histologic features of patients with different HCV subtypes within each major HCV type. In particular, patients infected with HCV types 1a and 1b had similar profiles in all of the variables examined. We wish to emphasize that the duration of HCV infection was estimated on the basis of clinical history, and the data on the correlation of estimated duration of HCV infection with HCV genotype should be interpreted with caution. Several differences were found between patients infected with different HCV types. A higher proportion of patients infected with HCV type 1 than of patients infected with other HCV types had acquired HCV through the transfusion of blood products. It has been suggested that HCV type 3 infection is more prevalent among intravenous drug users in Scotland (29). However, for the 16 patients in our study with HCV type 3 infection, intravenous drug use was not the predominant method of acquisition. This suggests that the association between HCV type 3 infection and intravenous drug use may not apply in the United States.

Patients with HCV type 3 infection were younger than patients infected with HCV types 1 and 2, and patients infected with types 3 and 4 had a shorter estimated duration of HCV infection than patients infected with type 1, as assessed from clinical history. There are three possible explanations for these observations. First, HCV types 3 and 4 may have been introduced into the United States more recently. Second, types 3 and 4 may be transmitted primarily

within younger generations through intravenous drug use. Third, there might be a bias in the estimation of HCV infection in patients infected with different HCV genotypes. That HCV type 3 is more prevalent in India and Scotland and type 4 is more prevalent in the Middle East favors the first explanation. In contrast, the observation that the proportion of patients who acquired HCV through intravenous drug use is higher for patients with types 3 and 4 than for patients with type 1 favors the second hypothesis. However, we must emphasize that a significant proportion of patients with HCV types 3 and 4 did not acquire HCV through intravenous drug use, which suggests that other factors are involved. In fact, these two possibilities are not mutually exclusive, and both may be operative.

An important observation in our study was that disease activity did not differ among patients infected with different HCV genotypes. A previous study in fewer patients (20) suggested that HCV type 2 infection was associated with a higher level of disease activity. Given the absence of an association in this larger, present study, it is possible that the previous association was the result of a type II statistical error. Nousbaum and colleagues (33) reported that patient age and presence of cirrhosis were independently associated with HCV type 1b infection in France and Italy. There are at least three possible explanations for the difference between their data and our observations. First, they had only 4 patients with HCV type 2 and 20 patients with HCV type 3, and thus a type II statistical error may have existed. Second, their patients with HCV type 1b infection were older and had longer estimated durations of disease compared with their patients infected with HCV types 2 and 3. Types 2 and 3 may have been introduced more recently to France and Italy and, therefore, the effect of these HCV genotypes on liver disease may not have been obvious at the time the study was done. Third, a significantly higher proportion of patients with HCV type 1 had acquired HCV through blood transfusion. Other clinical or epidemiologic factors may have been associated with the development of liver disease in patients with HCV type 1b infection. As discussed earlier, the distribution of HCV genotypes in patients receiving liver transplants was similar to that detailed in our current study, and this further supports the hypothesis that all HCV genotypes are equally able to induce active and potentially progressive liver disease.

With appropriate adjustments for an accurate assessment of viremia in different genotypes, we found that patients chronically infected with HCV types 1, 2, and 3 had similar levels of viremia. Similar results were recently seen in a large study of blood donors infected with different HCV genotypes (27). In contrast, in our study, the few patients with HCV type 4 had a lower level of viremia than patients with HCV type 1. The clinical and virologic implications of this low-level viremia in patients with HCV type 4 infection remains to be established.

Finally, it has been suggested that patients infected with HCV types 2 and 3 have a better chance of developing a long-term biochemical response to interferon-α (33–36). The patients in group 2 in our study received a complicated dose-adjustment protocol, and patients switched treatment arms if they did not show a complete biochemical response after 12 weeks of interferon-α therapy. Hence, our analysis of response to interferon-α therapy is complicated and is based on different criteria. These data, together with the treatment response data that involved the effect of changing interferon-α dose, will be addressed in a separate manuscript (Lindsay KL and colleagues. In preparation).

In conclusion, this line probe assay is applicable to patients in the United States who have chronic HCV infection. Type 1 is the most common HCV genotype in patients with chronic hepatitis C who are referred to tertiary centers in the United States. Half of patients with HCV type 1 have acquired HCV through the transfusion of blood products. No difference in disease activity or viremia levels was seen among patients chronically infected with HCV types 1, 2, or 3. The distribution of various HCV genotypes in other clinical settings (such as blood donors seropositive for anti-HCV and patients with chronic HCV infection seen by primary care doctors) and the relation between HCV genotypes and response to interferon therapy remains to be established.

Appendix

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ORIGINAL ARTICLE

Prevalence of Hepatitis C Genotypes among Patients with Chronic Hepatitis C in Norway

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Among 116 patients with biopsy-confirmed chronic hepatitis C (Riba 2 or Riba 3 positive) in a multicenter study in southern Norway on interferon, we determined hepatitis C virus genotype by restriction fragment length polymorphism (RFLP) of the 5′ NCR. The RFLP method was supplemented by and compared with a serological typing method based on the detection of type-specific antibody to peptide from the NS-4 region. A total of 102/106 (96%) patient sera showed detectable type-specific antibody to NS-4 peptides and corresponded in all cases, except two, to the genotype detected by polymerase chain reaction. Combining the results from RFLP genotyping and serotyping, genotype 1 was found in 40 (35%) (27 with 1a and 10 with 1b, 3 subtypes not determined), genotype 2 in 15 (13%) (subtype 2b in 14 and 1 subtype not determined), and genotype 3 in 58 (50%) of patients. The low mean age of the patients (34 years), the low prevalence of cirrhosis (3.5%), the short duration of the disease, and a high prevalence of intravenous-drug abusers may account for the low prevalence of infection with genotype 1b (9%). The epidemiological features of hepatitis C patients are markedly different from patient groups described in southern Europe in terms of risk factors, age, and genotype distribution.

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INTRODUCTION

Hepatitis C virus (HCV) exists as multiple, distinct genotypes. To date, six major genetic groups and at least 30 subgroups have been identified by analysis of complete or partial HCV genomic sequences (1). The genotype distribution has been found to be different in blood donors compared with patients with liver disease (2), and in intravenous drug abusers compared with other infected groups (3). A number of studies have shown that genotype 1 is widely distributed, being the predominant genotype in both the USA and Europe, particularly type 1b. Type 2 is also widely distributed around the world, and type 3 occurs with a prevalence of 5–42% in Europe. Type 4 shows a high prevalence in central Africa and Egypt (4), and type 5 is dominant in southern Africa (4, 5). Genotype 6 was found in up to 20–30% of patients in Vietnam and Hong Kong (6, 7), and HCV variants described as types 7, 8, and 9 have recently been identified in Vietnam (6) and Thailand (8). There is evidence to support the hypothesis that genetic variability of HCV is clinically significant (3). Many studies have shown that genotype 1b is an independent prognostic factor for a lack of response to interferon treatment (1, 3). In Scandinavia, Widell et al. (9) found a high prevalence of genotype 1a (57%) among Swedish blood donors, while Shev et al. (10) found a low prevalence of type 1b (10%). We therefore studied the genotypes of Norwegian patients with chronic hepatitis C in a multicenter interferon study by means of restriction fragment length polymorphism (RFLP) analysis. The results obtained with this method were compared with a serological assay based on the detection of type-specific antibody to NS-4 peptides.

MATERIALS AND METHODS

A total of 116 patients (85 male, and 31 female; median age 34 years, range 19–66 years) with biopsy-confirmed chronic hepatitis C (all were HCV antibody positive as assessed using Ortho's 2nd generation anti-HCV test (EIA-2) and RIBA2 or 3) were included in a multicenter interferon study (Roceron, A.F. Hoffman-La Roche Ltd, Basel). Risk factors for acquisition of HCV were previous intravenous drug abuse in 74 patients (64%), sporadic or unknown causes in 37 patients (32%), and blood transfusion in 5

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Table I. *Detection of type-specific antibody in serum samples of HCV-infected patients (106 known genotypes, 9 unknown)*

Genotype[a]	Serotype[b] 1	2	3	4	6	NTS	NR	Total	Sensitivity[c] (%)	Concordance[d] (%)
1a	26						1	27	96	100
1b	7				1	1	1	10	80	87.5
2b		14						14	100	100
3		1	52				1	54	98.1	98.1
4				1				1	–	–
Unknown	3	1	4			1	0	9	–	–
Total	36	16	56	1	1	2	3	115	95.7	98.2

[a] Determined by RFLP of 5′ NCR sequences.
[b] Detection of type-specific antibodies by ELISA to HCV genotypes 1–6. NR, non-reactive; NTS, non-type-specific antibody reactivity to NS-4 peptides.
[c] Number typeable by ELISA/number tested.
[d] Number concordant with genotype determination by RFLP/number typeable by ELISA.

patients (4%). Liver biopsy was performed within 1 year prior to the start of treatment. Histological evaluation was performed by one pathologist who had no knowledge of the clinical course or the biochemical and virological values. Chronic persistent hepatitis was found in 58 (50%), chronic active hepatitis with minimal activity in 26 (22%), chronic active hepatitis in 24 (21%), cirrhosis in 4 (3.5%), steatosis and fibrosis in 1 patient each, and 2 biopsies were too small for a definite diagnosis. The mean Knodell index was 5.5 ± 2.6 (range 3–11). Hepatitis C genotypes were identified by RFLP of the 5′ NCR, and serotyping was performed using NS-4 peptides to identify type-specific antibody to HCV (11, 12). The HCV viral load was analyzed according to Amplicor Monitor (Roche Diagnostics Systems, F Hoffman La-Roche Ltd, Basel, Switzerland) (13). Hepatitis B_s antigen was negative in all patients.

Statistics

Results are given as medians and ranges. Categorical variables were compared using the χ^2 test or the Kruskal–Wallis 1-way ANOVA. Comparison between two groups was done using the Mann–Whitney *U* test.

RESULTS

RFLP genotyping revealed type 1 in 37 patients (32%) (1a in 27 and 1b in 10), type 2b in 14 (12%), type 3 in 54 (47%), type 4 in 1 (1%). In 9 patients (8%) the RFLP were negative and the initial serum sample from 1 patient was missing. Detectable type-specific antibody to NS-4 peptides was demonstrated in 102/106 (96.2%) patients. In 9 of the patients with RFLP-negative samples, serotype 1 was found in 3 patients, serotype 2 in 1, serotype 3 in 4, and in 1 patient a non-type-specific antibody reactivity to NS-4 was found. In all cases except 2 (98.1%), the serotypes corresponded to the genotype detected by the RFLP assay (Table I).

Combining the results of the RFLP genotyping and the serotyping, genotype 1 was found in 40 patients (34%) (10 of genotype 1b), genotype 2 in 15 (13%), genotype 3 in 58 (50%), and genotype 4 in 1 patient.

The sex distribution and the median age was similar in patients with genotypes 1, 2, and 3 (34, 36, and 32 years, respectively; $p = 0.40$). Genotype 3 was marginally more prevalent among intravenous drug abusers compared with sporadic cases (55% versus 38%, $p = 0.051$). Patients with more advanced liver disease, such as moderate or severe chronic active hepatitis (CAH), were evenly distributed among the different genotypes. Analysis of variance showed a significant difference concerning baseline serum ALAT between genotypes 1, 2, and 3 (ALAT = 111, 120, and 161 U/l, respectively; $p = 0.02$). However, the baseline viral load in the different genotypes was not significantly different (395,000, 270,000 and 165,000 copies/ml for genotypes 1, 2, and 3, respectively; $p = 0.08$).

DISCUSSION

Studies from Europe (3) and the USA (13) have shown that the most common HCV genotype is 1b, with a prevalence of 50–70%. In contrast, in the present study from Norway only 9% of patients were infected with this genotype, with genotype 3 accounting for most cases of chronic HCV. There are several possible reasons for these discrepancies. Most of our patients were relatively young (median age 34 years) and only 4 had cirrhosis. The median duration of known disease in our study was 4 years, which is much shorter than in many other studies from Europe. In one study (3) the prevalence of genotype 1b was around 80% in subjects aged above 60 years or with HCV infection lasting for more than 15 years. Nearly two-thirds of our patients were former intravenous drug abusers, and some studies have shown a higher prevalence of genotype 3 in these patients (3).

Of the 115 patients, 106 were RFLP positive, and all but 1 were Amplicor reacative. This distinction may indicate that the RFLP was less sensitive than the Amplicor Monitor assay, probably due to the different detection systems used. The variation may also be due to improper sample handling. The distinction between genotypes 1a and 1b by RFLP is based on the difference in a single nucleotide.

Sporadic cases tended to be due to type 1, whereas type 3 was predominant among drug abusers ($p = 0.051$). The reason for this is not obvious. Perhaps these cohorts were infected not only by different routes, but also at different times and places. Drug abuse may have been introduced relatively late in Norway and a rapid spread of imported type 3 may have occurred recently.

We found no significant difference of viral load between genotypes 1, 2, and 3. This supports the hypothesis that viral load and genotype are independent predictors of reponse to treatment with interferon.

We found almost complete agreement between the results of serotyping with NS-4 peptides and typing with RFLP, as described recently (14, 15). This is of practical importance, since serotyping may be performed in most laboratories using an enzyme-linked immunosorbent assay (ELISA) and this method is now commercially available in Europe and elsewhere.

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fluence of Viraemia and Genotype Upon Serological eactivity in Screening Assays for Antibody to epatitis C Virus

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etection of antibody to recombinant proteins erived from hepatitis C virus (HCV) genotype 1 epresents the principal method for diagnosis of CV infection. A method was developed for uantifying antibody reactivity in two third-gen- ration enzyme immunoassays (Ortho EIA 3.0 nd Murex VK48), and the influence of viraemia, CV genotype, and host factors such as age, ender, and risk group upon antibody levels ere investigated in a consecutive series of 117 nti-HCV-positive volunteer blood donors. Vi- aemic donors (as assessed by the polymerase hain reaction; PCR) showed significantly higher evels of anti-HCV by the Ortho EIA than those ho were nonviraemic (adjusted mean differ- nce of 10.1 fold after multiple regression analy- s). The only other factor to influence signifi- antly antibody level was genotype, where it was ound that donors infected with type 1 showed 4 o 4.5 times greater serological reactivity by the rtho assay than those infected with type 2 or 3. ntibody levels by the Ortho assay correlated osely to those detected by the Murex VK48 as- ay, and similar differences between PCR-posi- ve and negative donors and between those in- ected with different genotypes were found. ifferences in serological reactivity between enotypes indicate that a large proportion of pitopes of the type 1a or 1b recombinant pro- eins used in current assays are genotype spe- fic. Variation in sensitivity of screening assays or different genotypes is of potential concern hen used in countries where non-type 1 geno- pes predominate in the blood donor or patient opulation.

EY WORDS: blood donor, screening, ALT, antigenic variation

INTRODUCTION

Following the identification of hepatitis C virus (HCV) as the principal aetiological agent of posttransfusion non-A, non-B hepatitis [Kuo et al., 1989; Choo et al., 1989], many countries have established routine screening of all blood donors for anti-HCV-specific antibody by enzyme immunoassay (EIA). Most commonly used assays use recombinant proteins derived from the prototype HCV clone, HCV-PT (genotype 1a). However, other variants of HCV may differ substantially in nucleotide sequence from one another and show varied geographical and epidemiological distributions [Bukh and Miller, 1994; Simmonds, 1995]. In particular, the inferred amino acid sequences of the envelope glycoproteins and nonstructural proteins differ considerably, leading to the possible existence of type-specific as well as shared epitopes between genotypes. It remains unclear whether such assays are equally effective for detecting antibody elicited by heterologous genotypes.

Previous investigations of serological reactivity to individual HCV proteins by immunoblotting assays indicated type-specific reactivity to nonstructural proteins used as antigens in current screening enzyme-linked immunosorbent assays (ELISAs). For example, reactivity to c100-3 and 5-1-1 antigens used in the original first-generation screening assays for HCV was found more frequently in blood donors infected with HCV type 1a or 1b (90%) than in those infected with type 2 or 3 [30–35%; Chan et al., 1992; McOmish et al., 1993]. Although second and third-generation screening assays for antibody to HCV contain a wider range of antigens, the frequency and strength of serological reactivity to each component have also been shown to vary between genotypes. For example, individuals in-

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Study was undertaken at University of Edinburgh.

cted with type 3 were statistically more likely to show ıdeterminate results by second-generation recombi-ant immunoblot assay (RIBA-2; Chiron, Emeryville, A) generally reacting with only the highly conserved 22-3 protein (90% amino acid sequence similarity be-veen genotypes), and not to the more variable non-ɜructural proteins NS-3 and NS-4 [McOmish et al., 994].

Although differences in serological reactivity exist etween HCV genotypes, it has proved extremely diffi-ılt to obtain direct information on whether this is ıfficient to reduce significantly the effectiveness of ırrent screening assays for anti-HCV used for blood ɔnor screening. This is partly because analyses of sero-ɔgical reactivity have so far been confined to samples ıat have already been identified as anti-HCV positive. ı addition, no practical antibody assay has been pro-ıced that is based upon a different HCV genotype so reventing a comparison with conventional assays. ther methods of detection of infection that are inde-endent of serology, such as the polymerase chain reac-on (PCR), are impractical for use on large numbers of lood donor samples.

In the current study a method was developed to quan-fy levels of antibody to HCV antigens using two com-ıercial assays (Ortho EIA 3.0 [Ortho Diagnostic Sys-ems, Raritan, NJ] and Murex VK48 [Murex, Dartford, ent, U.K.] currently employed for blood donor screen-ıg amongst blood donors infected with different geno-ɣpes. Both assays are third generation and contain ntigens corresponding to regions within the core, S-3, NS-4, and NS-5 proteins encoded by viruses of enotype 1a (Ortho) or 1b (Murex). Quantitation of an-body levels allowed a detailed analysis of the factors ıat influenced serological reactivity to genotype 1 re-ɔmbinant proteins, including viraemia status, geno-ɣpe, and donor factors such as age, risk factors for ıfection, and gender.

MATERIALS AND METHODS

Donor Samples

Aliquots of plasma were obtained from consecutive cottish blood donors who were repeatedly reactive by econd or third-generation anti-HCV screening using bbott, Ortho, or Murex assays. A total of 117 study amples were selected for the study according to the llowing criteria: a) Samples were either confirmed by IBA-2 (n = 108), or were RIBA-2 indeterminate but CR positive (n = 9; all type 3). b) For each sample sed, it was possible to recall the blood donor and ob-ain a range of baseline data, such as age, gender, risk ıctor(s) for infection, alanine aminotransferase level, nd geographical origin.

HCV Genotyping

HCV genotypes were determined by a serological ɣping assay based upon the detection of antibody to ɣpe-specific epitopes in the NS-4 region [Bhattacherjee , al., 1995]. This assay shows high sensitivity and xcellent concordance with genotyping assays such as restriction fragment length polymorphism (RFLP) assays in this blood donor population and in patients with chronic hepatitis [Lau et al., 1995; Bhattacherjee et al., 1995]. The majority of samples used in the current study had been the subject of a previous comparison with an RFLP genotyping assay, and showed concordant results in 121 of the 122 samples that could be tested by both methods [Simmonds et al., 1993]. The single discrepant sample (type 3 by RFLP, type 1 by the serological genotyping assay) was not used in this study. The advantage of the serotyping assay was its ability to determine genotype in samples that were PCR negative (22 of the 116 donations described here; Table I), although in these there can by definition be no corroborating evidence from genotyping assays that the genotypes were correctly identified. However, inclusion of these results allowed the contributions of viraemia and genotype to be assessed simultaneously by multiple regression analysis (see below).

TABLE I. Genotypes, Viraemia Status, and Background of the Study Group

	HCV genotype[a]			
	1	2	3	Total[b]
Total	50	15	52	117
Viraemia status				
PCR+	37	13	44	94
PCR−	13	2	8	23
Gender				
Male	27	10	34	71
Female	20	2	15	37
Risk group				
IVDU	20	3	21	44
Other[c]	16	6	15	37
Unknown	11	4	8	23
Age range[d]				
20–29	20	1	18	39
30–39	17	3	20	40
40–49	6	7	5	18

[a]Genotype determined by serotyping assay [Bhattacherjee et al., 1995].
[b]Background information was not available from a small, variable proportion of donors, so that this column does not always add up to 117.
[c]This category includes previous transfusion (n = 17); tattooing/other parenteral exposure (n = 9), IVDU contact (n = 10), and family contact (n = 1).
[d]Treated as a continuous variable by multivariate analysis.

Viraemia was assessed by PCR as described previously [Chan et al., 1992]. 0.5 ml aliquots of plasma was ultracentrifuged at 100,000*g* for 2 hours at 4°C and RNA was extracted from the pellet by incubation in proteinase K/Sarkosyl prior to phenol/chloroform extraction, ethanol precipitation, and resuspension in 20 μl nuclease-free water. Five microlitres of RNA was reverse transcribed using an antisense primer (209) specific for the 5′ noncoding region (5′NCR), followed by amplification using primers 209 and 939 for the first round and 211 and 940 for the second, nested PCR. This method has an overall sensitivity of 200 copies of RNA/ml.

Antibody to individual antigens c22-3, c33c, c100-3, and 5-1-1 was assayed using RIBA-2 (Chiron) in accordance with the manufacturer's instructions.

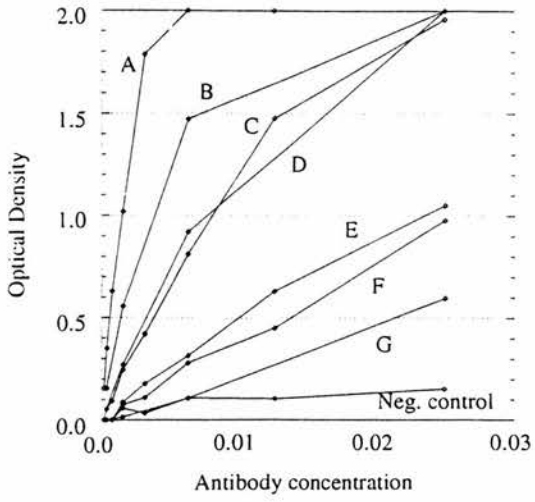

Fig. 1. O.D.s of anti-HCV-positive plasma samples (A-G) and an anti-HCV-negative control assayed at different dilutions in the Murex VK48 EIA. Sample dilutions expressed as concentration (e.g., a 1/40 dilution corresponds to a concentration of 0.025, 1/160 to 0.00625).

Measurement of Antibody Levels

Quantitation of antibody by the Ortho third-generation EIA (Ortho 3.0 EIA) and Murex VK48 EIA was carried out by assay of multiple dilutions (in anti-HCV-negative plasma) of samples as described in Results. In all other respects, each EIA was carried out in exact accordance with the manufacturer's instructions. To maintain consistency, the same anti-HCV-negative plasma sample was used for all dilutions. The positive control was another large volume plasma sample from an HCV type 1a-infected individual that was assigned a nominal antibody concentration of 1.0 unit, and which was used in all assay runs.

Because of the linear relationship between optical density (O.D.) and concentration of sample added to each well (over the range from 0 to 1.0; Fig. 1), anti-HCV reactivity could be quantified in terms of O.D., provided each sample was diluted sufficiently to produce an O.D. in the linear range of the assay. Each EIA plate included replicate dilution series of the reference sample, providing at least four absorbance values (two for each dilution) within the linear range of the assay. Antibody levels in test samples were measured relative to the mean value obtained from the reference sample on the plate on which they were tested. This controlled for the observed minor variation between plates and between batches in the performance of the EIA (±10–20% in absorbance values). For calculation of antibody levels, the O.D. value obtained from the negative control (also used as diluent for the test and reference specimens), representing nonspecific binding to the wells, was subtracted from both test and reference O.D.s. Insufficient volumes were available to measure antibody levels for one sample in the Murex VK48 assay and for seven samples in the Ortho EIA 3.0.

This method was chosen over titration to an end-point as it has advantages of accuracy, and controls for the effect of plate-to-plate variation. However, because of the linear relationship between concentration of sample added and absorbance, it would be possible in principle to convert each antibody level measured in this study to a dilution at which an O.D. assigned as the cut-off value (e.g., 0.1) would be obtained. This would allow results to be expressed as end-point titres. An analysis of several different methods for antibody quantitation has been presented elsewhere [Simmonds, 1987].

RESULTS

Measurement of Antibody Levels

Over a wide range of values, O.D.s by the Murex screening assay were proportional to the concentration of anti-HCV-positive plasma added (Fig. 1). Although the concentration of antibody within each specimen varied, a linear relationship was found between O.D. values of 0 to 1.0. For the following calculations we have assigned sample C as the reference antiserum with a nominal antibody level of 1.0 unit. Antibody levels in other samples relative to C can be calculated using the following formula:

$$\text{Test antibody level} = \frac{\text{Test O.D.} \times \text{reference concentration}}{\text{Ref. O.D.} \times \text{test concentration}}.$$

For example the O.D. of sample E is 0.632 at a concentration of 0.0125 (dilution of 1/80), while the reference sample has an O.D. of 0.812 at 0.00625. Therefore, sample E has a level of $(0.632 \times 0.00625)/(0.812 \times 0.0125) = 0.39$ arbitrary units of antibody (or about 4/10 the level in C). Because of the proportionality between O.D. and antibody concentration, essentially the same results can be obtained by selecting different test or reference dilutions. For example, the antibody level of sample E was 0.38 using the O.D. at a concentration of 0.00625 (O.D. = 0.315). Using the above formula the amounts of anti-HCV antibody for the samples are as follows: A) 3.1 units; B) 1.4 units; C) 1.0 units; D) 0.67 units; E) 0.38 units; F) 0.28 units; G) 0.18 units. A similar linear relationship between antibody concentration and O.D. was observed upon titration of the same samples in the Ortho EIA 3.0 (data not shown).

Clearly, the most accurate results are obtained using dilutions of antibody that produce O.D.s of at least 0.2 and less than 1.0, beyond which point the linear relationship with antibody amount was lost (Fig. 1). These limits were applied to antibody level measurements for all samples by both assays in this study.

Study Group

Approximately 80% of samples from the study group were HCV RNA PCR positive and the frequency of viraemia was similar amongst the three HCV genotypes detected in this population (74% type 1; 87% type 2; 85% type 3). Genotype distributions varied in male and

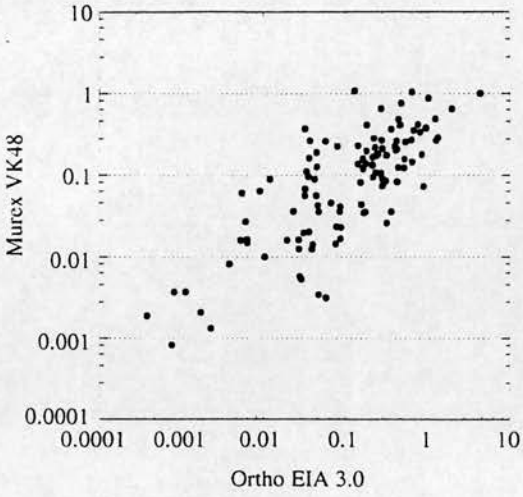

Fig. 2. Comparison of antibody levels in the Ortho EIA 3.0 and Murex VK48 EIAs for the study samples, plotted on a $\log_{10}$ scale.

emale donors (males accounted for 57%; 83%; 70% of nfections with types 1, 2 and 3 respectively), and there as under-representation of type 2 amongst donors in hom drug abuse was identified as the principal risk actor (3/13 for type 2 compared with 20/47 and 21/23 or types 1 and 3). Possibly related to this observation is he marked difference in age distribution between type infected donors (median 41 years) compared with type (30 years) and type 3 (30 years).

Antibody Levels in Blood Donor Samples

Each of the study samples was assayed by both Ortho nd Murex screening EIAs at a series of dilutions (usu-lly 1/40, 1/160, and 1/640) to measure antibody level elative to the reference sample. Values ranged from .0007 to 3.5 in the Ortho assay (a 5,000-fold range), nd from 0.0008 to 1.02 in the Murex assay (range ,275-fold). A close correlation was found between anti-ody levels measured in the two assays (Fig. 2), which as highly significant by regression analysis ($r = .787$; $P = 0.0001$).

Analysis of Factors Influencing Antibody Levels

The levels of circulating antibody detected by the two creening assays could be influenced conceivably by a ide range of virus and host-specific factors. These in-lude viraemia, HCV genotype (see Introduction), route f infection/risk group, donor age, and gender. With the rtho assay, the distribution of antibody levels differed etween samples that were PCR positive and negative Fig. 3A) and also between genotypes (Fig. 3B). There as a 20-fold difference in the median antibody level or PCR-positive samples (0.215), compared with PCR egatives (0.010), and a similar difference was found or the Murex VK48 assay (0.126 compared with 0.013; ig. 3E). A similar difference in antibody levels by both ssays was also detected between different genotypes Fig. 3A,D). In the Ortho assay, median antibody levels ere approximately fivefold higher in type 1-infected donors compared with those infected with type 3 (medians 0.275 and 0.056, respectively), similar to the difference observed using the Murex assay (medians of 0.185 and 0.065).

There was no significant differences in antibody levels by either assay between males and females (data not shown), age (Fig. 3C,F), or risk group (data not shown). Thus, there was no evidence from this preliminary analysis that these host factors could account for the observed differences in antibody level between genotypes or between PCR positives and negatives.

In order to investigate the relative contribution of viraemia and genotype to antibody levels, and the extent to which these are influenced by other factors, we carried out multiple regression analyses of both assays fitting genotype, risk group, gender, and age as covariates. This allowed the size and significance of differences between groups to be calculated and controlled by other factors.

By this analysis, viraemia and genotype proved to be the principal influences upon antibody level by both assays. Adjusted antibody levels by the Ortho assay were 10.2 times higher in viraemic donors, and between 4 and 4.5 times higher in type 1-infected donors compared with genotypes 2 and 3. Similar although less marked differences between these categories emerged from comparison of antibody levels by the Murex VK48 assay. The only other comparison that approached significance was the observed higher levels of antibody by the Murex assay in intravenous drug abusers (IVDUs) compared with donors infected by other parenteral routes. No difference, however, was found by the Ortho assay. Age was not correlated with antibody level in either assay ($P = 0.52$, $P = 0.33$, respectively; n.s.).

Alanine Aminotransferase (ALT) Levels

Although antibody levels were the principal outcomes investigated in this study, ALT levels were investigated as an alternative outcome of these host factors. Multiple regression analyses of ALT fitting genotype, risk group, gender, and age as covariates were carried out. The only significant influence upon ALT levels was viraemia, where PCR-positive donors showed an adjusted mean ALT level approximately 20 times higher than PCR negatives. With adjusted data there was no evidence for a difference in ALT levels between genotypes, risk group, gender, or age (Table II; age: $P = 0.70$).

DISCUSSION

The main influences upon anti-HCV antibody level as measured by either Ortho or Murex assays in this study were viraemia and genotype, with significantly higher levels being found in donors who were PCR positive and infected with genotype 1. The detection of circulating virus by PCR indicates the presence of active HCV infection, and the higher antibody levels observed in this group may result from persistent stimulation of the immune system. This explanation is consistent with the finding of an association between

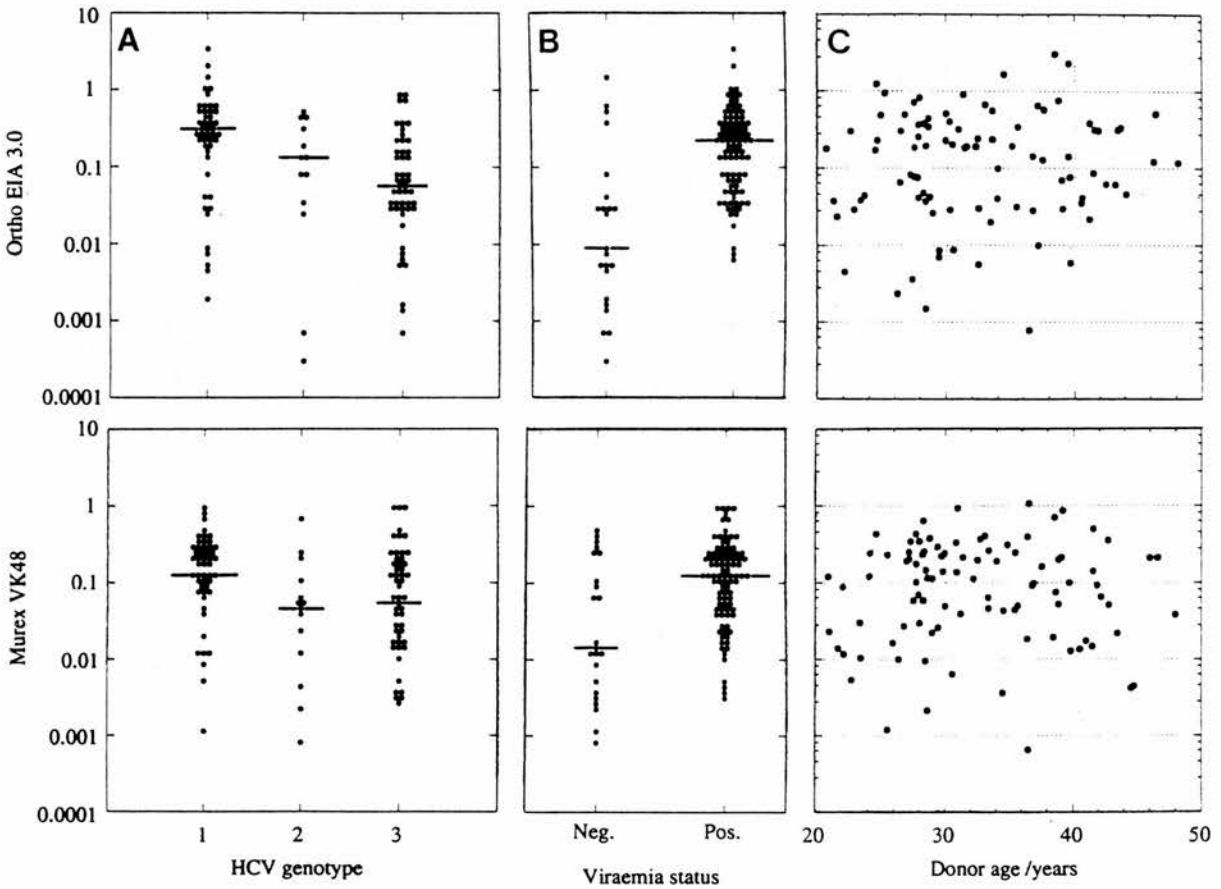

Fig. 3. Distribution of antibody levels in Ortho EIA 3.0 (**A, B, C**) and Murex VK48 EIA (**D, E, F**) in study group categorised for viraemia status (A, D), genotype (B, E), and age (C, F). Median values indicated by horizontal bar.

TABLE II. Adjusted Influence of Viraemia, Genotype, and Host Factors Upon a Antibody and ALT Levels

	Ortho EIA 3.0		Murex VK48		ALT	
Factor[a]	Adjusted ratio[b]	*P* value*	Adjusted ratio	*P* value*	Adjusted ratio	*P* value*
Viraemia						
Positive-negative	10.2	<0.00001	3.54	0.0011	0.053	0.032
Genotype						
Type 1-type 2	4.12	0.005	3.72	0.014	0.97	0.95 (n.s.)
Type 1-type 3	4.48	<0.00001	2.35	0.0070	0.84	0.47 (n.s.)
Type 3-type 2	0.93	0.88 (n.s.)	1.58	0.38 (n.s.)	1.16	0.76 (n.s.)
Gender						
Female/male	1.06	0.83 (n.s.)	1.63	0.13 (n.s.)	0.68	0.12 (n.s.)
Risk						
IVDU-other	1.22	0.52 (n.s.)	2.31	0.049	0.84	0.50 (n.s.)
IVDU-unknown	1.24	0.59 (n.s.)	1.81	0.075 (n.s.)	1.03	0.93 (n.s.)
Other-unknown	1.01	0.97 (n.s.)	1.28	0.57 (n.s.)	1.23	0.55 (n.s.)

[a] Age is a continuous variable and cannot be represented in the table (see text).
[b] Adjusted ratio of antibody or ALT levels after multiple regression analysis between the first and second category (e.g., PCR positive compared with PCR negative).
* Significant *P* values (<0.05) are underlined; nonsignificant values indicated (n.s.).

viraemia with ALT abnormalities, presumably reflecting a link between virus replication and liver damage. Amongst the PCR-negative group, most had ALT levels within the normal range for blood donors (0-55 IU/ml), suggesting low levels of virus replication or complete clearance of HCV.

In this study we sought to investigate the extent to which antigenic variation between genotypes affects

e sensitivity of current screening assays. Lower sero-gical reactivity was found to the type 1a or 1b anti-ens used in the EIAs with samples from donors in-cted with non-type 1 genotypes. The study was esigned to examine the separate influences of host ctors that might compound the observed differences etween genotypes in serological reactivity. By multi-ariate analysis, no evidence was found for any signifi-nt effect of age, risk group, or gender upon antibody activity, while the genotype differences were shown be independent of the differences in antibody levels etween PCR-positive and -negative samples (see bove). The magnitude of the difference in levels pro-des an estimate of the relative amounts of genotype-ecific and cross-reactivity antibody to the core, NS-3, S-4, and NS-5 antigens. For example, the 4-4.5-fold fference between type 1 with types 2 and 3-infected nors could be interpreted as indicating that a major roportion of antigenic determinants in the EIAs are enotype specific. This conclusion is consistent with the nding of type-specific epitopes in core, NS-4, and NS-5 gions [Machida et al., 1992; Simmonds et al., 1993; anaka et al., 1994; Bhattacherjee et al., 1995; Zhang al., 1995]. Although detailed mapping of type-spe-fic epitopes in the NS-3 and NS-5 regions used in reening assays has not been published, the existence significant amino acid sequence variability in both of ese regions predicts the existence of substantial anti-enic variation that may also contribute to the ob-rved differences in this study.

This analysis cannot rule out completely the possibil-y that type 1 infections elicit higher levels of antibody an other genotypes, although if this were the case en it would be expected that type 1 infections would associated with a higher mean ALT level if antibody vel was related to a greater severity of disease. This is early not the case, as it was found that adjusted mean LT values were within 16% of each other (Table II). milarly, although there is abundant evidence that pe 1-infected patients are less likely to respond to terferon treatment than types 2 and 3, evidence for a fference in pathogenicity between genotypes has oved more elusive. A similar spectrum of liver disease as been described in patients infected with each of the enotypes, with little evidence that type 1 is more patho-enic once the effect of age (and duration of infection) is ken into account. Recently, it has been shown that ean levels of viraemia are similar between genotypes 1, and 3, once the greater sensitivity of the bDNA assay r type 1 sequences is taken into account [Smith et al., in ess; Lau et al., submitted], effectively discounting any herent difference in replicative capacity or immunolog-al control between them in vivo. Consistent with these ew data is the previous observation that multiple-ex-sed individuals such as haemophiliacs do not show an errepresentation of any particular genotype compared ith the distribution of genotypes in the plasma from hich the blood products were manufactured [Jarvis et ., 1994], indicating that type 1 in particular shows no ndency to replace other genotypes upon reinfection.

This study provides grounds for concern that antigenic variability of HCV impairs the performance of current assays. For example, if the current assays were 4.5 times less sensitive than they currently are (the difference between antibody levels of type 1 and 3 donors; Table II), then it is possible that a proportion of samples currently detected by screening assays would become negative. We have retested samples from the 50 type 1-infected donors in the Ortho EIA at a 1/90 dilution compared with the recommended 1/20 dilution (therefore making the assays equivalent to their current sensitivity for type 3-infected donors), and observed a decrease in O.D.s for many of the samples. Of interest was the observation that one RIBA-confirmed sample became borderline at the 1/90 dilution (O.D. 0.69; assay cut-off 0.65). Further studies are required to investigate the clinical significance of these differences in sensitivity.

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Use of NS-4 peptides to identify type-specific antibody to hepatitis C virus genotypes 1, 2, 3, 4, 5 and 6

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The 5′ end of the NS-4 protein of different genotypes of hepatitis C virus (HCV) is highly variable in nucleotide and inferred amino acid sequence, with frequent predicted amino acid substitutions between all six of the major HCV genotypes described to date. This region has been shown to be antigenic by epitope mapping, and elicits antibody in HCV-infected individuals with a detectable type-specific component. We have used this sequence data to specify branched peptides for an indirect binding/competition assay to detect type-specific antibody to each major genotype. A total of 183 out of 210 samples (87%) from blood donors and patients with chronic hepatitis C infected with genotypes 1 to 6 showed detectable type-specific antibody to NS-4 peptides that in almost all cases (> 97%) corresponded to the genotype detected by a PCR typing method. These findings demonstrate the existence of major antigenic differences between genotypes of HCV, and indicate how infection with different variants of HCV may be detected by a serological test.

Introduction

Hepatitis C virus (HCV) is the major causative agent of post-transfusion non-A, non-B hepatitis. The viral genome is a single-stranded, positive-sense RNA of about 9400 nucleotides in length (Choo *et al.*, 1991), encoding a single, continuous polyprotein that is cleaved into putative core, E1, E2/NS-1 (envelope), NS-2, NS-3, NS-4 and NS-5 (non-structural) proteins. Comparisons of published complete genome sequences have found that isolates fall into distinct virus types (Choo *et al.*, 1991; Kato *et al.*, 1990; Okamoto *et al.*, 1991; Takamizawa *et al.*, 1991; Okamoto *et al.*, 1992*b*; Sakamoto *et al.*, 1994). According to recent proposals (Simmonds *et al.*, 1994*a*), based upon extensive sequence comparisons of HCV in the core (Bukh *et al.*, 1994), E1 (Bukh *et al.*, 1993) and NS-5 regions (Simmonds *et al.*, 1993*a*), variants of HCV can be usefully classified into six major genotypes (designated 1 to 6), many of which can be further subdivided into a number of more closely related subtypes.

The variation in nucleotide sequences between HCV isolates is expressed in the translated amino acid sequences and may alter the antigenic properties of viral proteins. For example, sera from individuals infected with non-type 1 genotypes react infrequently with recombinant proteins derived from NS-4 (5-1-1 and c100-3) used in current screening and confirmatory assays for HCV (McOmish *et al.*, 1993; Chan *et al.*, 1991); there is also evidence for less frequent reactivity to the NS-3 protein (c33c) amongst those infected with types 2 to 6, leading to a statistically significant over-representation of 'indeterminate' results from these variants in confirmatory assays such as the 2nd generation recombinant immunoblot assay (RIBA-2) (McOmish *et al.*, 1994).

At present PCR-based methods are the principal means by which variants of HCV may be identified (Okamoto *et al.*, 1992*c*; Stuyver *et al.*, 1993*a*; Chayama *et al.*, 1993; McOmish *et al.*, 1994). The major drawbacks of PCR-based methods, however, are the expense and complexity of the procedure which makes difficult the processing of large numbers of clinical samples on a routine basis. Serological methods for HCV typing, based upon the ability to distinguish antibody elicited by

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infection by different genotypes of HCV are necessarily indirect, and not always appropriate (as they rely upon a humoral immune response in the infected individual), but do have important practical advantages over PCR-based methods in ease of use and cost. The problem with developing such tests lies in finding protein sequences which are both type-specific and antigenic.

Epitope mapping of the core and NS-4 proteins has revealed the existence of linear antigenic determinants in regions of variability between HCV genotypes (Tsukiyama Kohara *et al.*, 1993; Simmonds *et al.*, 1993*c*). ELISAs based on peptides corresponding to these regions have been used to identify type-specific antibody elicited by genotypes 1 and 2 (core or NS-4; Tsukiyama Kohara *et al.*, 1993; Tanaka *et al.*, 1994; Mondelli *et al.*, 1994), and 1, 2 and 3 (NS-4; Simmonds *et al.*, 1993*c*). In the latter assay, cross-reactivity due to epitopes shared between genotypes was minimized by co-incubation of serum with peptides in solution heterologous to those on the solid phase.

In this paper, we have obtained nucleotide sequences of the antigenic region of NS-4 for the more recently described genotypes of HCV (types 1c, 2c, 3b, 4a, 5a and 6a) and have developed a competition ELISA to detect type-specific antibody to each of the six genotypes. The frequency with which type-specific antibody to NS-4 could be detected was measured by testing sera from individuals infected with a range of different genotypes.

Methods

Samples. HCV sequences from the 5′ non-coding region (5′NCR) were amplified by PCR from plasma or serum samples from anti-HCV positive blood donors from Egypt, South Africa and Hong Kong. Genotypes were identified by sequence comparisons with previously published 5′NCR sequences for identification of genotypes (see Fig. 1). Sequences were obtained in the NS-4a region from those infected with genotypes 4, 5 and 6, as well as from samples from individuals previously shown to be infected with type 1c and 2c (Simmonds *et al.*, 1993*a*).

Investigation of the frequency of type-specific antibody to NS-4 peptides was carried out using a combination of anti-HCV positive blood donors ($n = 39$) and patients with HCV-induced liver disease ($n = 171$) infected with genotypes 1 to 6 as identified by RFLP of the 5′NCR as previously described (McOmish *et al.*, 1994; Davidson *et al.*, 1995).

PCR amplification of NS-4 sequences. RNA was reverse transcribed using an antisense primer with the sequence AACTCGAGTATCCCACTGATGAAGTTCCACAT (primer 007 from Simmonds *et al.*, 1993*c*) which is complementary to a conserved sequence from the HCV NS-4 coding region. The outer and inner sense primers for nested PCR amplification were designed from the two regions of greatest conservation towards the 5′ end of known NS-4 sequences of genotypes 1a, 1b, 2a, 2b and 3a. Complementary DNA was amplified in the first round using 007 in combination with sense primer CACCCCATCACCAAATACAT (5′ base: 4915; nucleotides numbered as in Choo *et al.*, 1991). The second round of PCR amplification used sense primer TTTT**GGATCC**ATGCATGTCAGCTGATCTGG (5′ base: 4941) and antisense primer TTTT**GGATCC**ACATGTGCTTCGCCCAGAA (5′ base: 5278). Both primers used in the second PCR step included terminal *Bam*HI restriction sites (in bold) to allow cloning via cohesive ends. The NS-4 sequence of genotype 6 was found to amplify better using a combination of primers that matched genotypes 1, 2 and 3 (Simmonds *et al.*, 1993*c*) for the first and second rounds of PCR followed by a third round employing primers 5351 and 594 to enable cohesive-end cloning.

Cloning of amplified NS-4 sequences. All cloning steps followed standard protocols (Ausubel *et al.*, 1992) using Boehringer Mannheim enzymes except where stated. The amplified NS-4 DNA bands ($\approx 0{\cdot}4$ kb) were excised from 1% agarose gels and the DNA was recovered by low-speed centrifugation through glass wool (Heery *et al.*, 1990). Extracted DNA was cleaved with *Bam*HI and ligated into *Bam*HI-digested pUC18 vector (Yanisch-Perron *et al.*, 1985) at 16 °C overnight, and used to transform competent *Escherichia coli* cells (XL1-Blue from Stratagene). Plasmid DNA from colonies containing inserts was sequenced with forward and reverse pUC/M13 primers using the United States Biochemical Sequenase kit. The manufacturer's protocol for double-stranded sequencing with alkaline denaturation was followed except that the reactions were carried out in 10% dimethyl sulphoxide.

Sequence analysis of NS-4. Phylogenetic analysis of NS-4 sequences was carried out with the programs DNADIST, NEIGHBOR and DRAWTREE in the PHYLIP package version 3.5c kindly provided by Dr J. Felsenstein (Felsenstein, 1993). Unrooted trees were constructed by the neighbour-joining algorithm as previously described for analysis of NS-5 sequences (Simmonds *et al.*, 1993*a*).

Synthesis of peptides for HCV serotyping assay. Synthetic peptides were prepared on *p*-hydroxymethylphenoxymethylpolystyrene (HMP) resin bearing a branched-lysine core to give eight copies of the same sequence per molecule, using the multiple antigenic peptide (MAP) system (Fmoc MAP 8-Branch; ABI Ltd; Tam, 1988). Synthesis was performed using Fastmoc chemistry on an Applied Biosystems model 432A automated peptide synthesizer (ABI Ltd). Purity of the peptides was determined by reverse-phase HPLC. Integration of peaks by computer analysis (PE Nelson, Perkin Elmer) showed all peptides to be greater than 80% pure. Peptides were not purified further prior to use in the assay.

Serotyping of serum samples using the 1–6 ELISA. Each peptide was reconstituted in distilled water at an approximate concentration of 1 mg/ml. A small volume of 30% acetic acid was added to the type 3 peptides to help them dissolve, while the remaining peptides dissolved more easily after bubbling ammonia vapour through the solution.

Polypropylene microtitre wells (Immunoplate, GIBCO BRL) were coated with 100 µl volumes of phosphate buffered saline (PBS) containing all 21 peptides, each at a concentration of 50 ng/ml. The plates were incubated at 4 °C overnight, washed in PBS and blocked with 125 µl volumes of blocking solution (PBS, 2% bovine serum albumin) for a minimum of 1 h at room temperature. After blocking, plates were air-dried and kept at 4 °C prior to use. Plates prepared in this way could be stored for several months without loss of reactivity.

Prior to the addition of the serum to the wells, blocking solutions of competing peptides (in a 100-fold excess over those used for coating) were added to the appropriate wells on the microtitre plate in volumes of 10 µl per well. Plasma, diluted in 800 µl of sample diluent (Murex Diagnostics) at a concentration of 1:20, was immediately added to each of the eight wells used per sample (100 µl per well). This was followed by incubation overnight at 4 °C.

Plates were washed four times in PBS + 0·05% Tween 20 (PBST), and then incubated with 100 µl of conjugate (Murex; VK 47 –

horseradish peroxidase-labelled mouse monoclonal antibody to human IgG) for 1 h at 37 °C. The plates were washed again, four times in PBST, before adding 100 µl substrate to each well (TMB, Murex) and incubating for 30 min at 37 °C. Sulphuric acid (8 M, 50 µl) was added to each well before reading the plate against a negative control blank at 450 nm, with a reference wavelength of 690 nm.

Statistical analysis. Analysis of the median and variance of the absorbance values recorded for plasma samples was carried out using standard statistical software (SYSTAT). Box plots represent the 25th and 75th quartiles of a non-parametric distribution as the boundaries of the box, and the median as the line dividing the box (Tukey, 1977). The whiskers represent the range of values that lie within 1·5 times the interquartile range (inner fence). Values between 1·5 and 3 times the interquartile range are plotted as asterisks (*), while open circles (○) represent outlying values greater than three times the interquartile range (outer fence). For calculation of median values and ranges of serological data, a value of 2·0 was substituted for samples with absorbance values greater than 2.

Results

Phylogenetic analysis of the NS-4 region

HCV types 4, 5 and 6 were identified by sequence comparison of the 5′NCR with those of published variants (Fig. 1). In order to obtain cloned NS-4 nucleotide sequences from these genotypes, RNA was reverse transcribed and amplified using conserved primers from the NS-4 region, as described in Methods. Sequences were obtained from six type 4a samples (EG-1, -13, -21, -24, -25 and -33), four type 5a (T478, SC-6, SC-23 and SC-24) and two type 6a (HK-4 and T3950). NS-4 sequences were also amplified from previously identified samples of genotypes 1c (4TY4), 2c (T983), 3b (B4, B9) identified by sequence comparisons in NS-5 (Simmonds *et al.*, 1993*a*) and unpublished data).

The NS-4 region used in the phylogenetic analysis covers the nucleotide sequence positions 4941–5282 (numbered as in Choo *et al.*, 1991) and is 341 bases in size. Previously published nucleotide sequences, and the corresponding regions of complete genomes (numbered 1–33; Fig. 2) were included in the comparison with the newly isolated NS-4 sequences (nos 34–50; Fig. 2). The unrooted phylogenetic tree displays three levels of branching which reflects different levels of variability between isolates. The major branches correspond to the six major genotypes of HCV based on analysis of NS-5 (Simmonds *et al.*, 1993*a*) and E1 sequences (Bukh *et al.*, 1993). The second level of branching occurs between variants of HCV previously described as subtypes (Simmonds *et al.*, 1993*a*). As found in NS-5 and E1, NS-4a sequences show discontinuous, 'hierarchical', as opposed to continuous, variation between isolates. The grouping of the individual isolates was in all cases consistent with genotype classifications obtained using the 5′NCR (Fig. 1), and E1 and NS-5 (Simmonds *et al.*, 1994*b*).

Analysis of the distribution of pairwise distances

The relationship between sequence diversity between major genotypes and subtypes was investigated by comparison of the distributions of evolutionary distances of separate pairwise comparisons between major genotypes, subtypes, and variants within a subtype (isolates) (Table 1*a*). The median nucleotide distance of comparisons between members of different major genotypes was 0·381, greater than those calculated for subtypes and isolates (0·168 and 0·037). Although the range of values of type, subtype and isolate overlapped, the 95th percentile ranges did not. The range of nucleotide distances corresponded closely to three discrete normal distributions, as previously described for NS-5 (Simmonds *et al.*, 1993*a*), as shown by the similarity of mean and median values, and between the actual 95th percentile ranges with those calculated from the mean ± 2 standard deviations (Table 1*a*).

The analysis was extended to the distribution of pairwise distances calculated between inferred amino acid sequences (expressed as the proportion of different residues). Such comparisons are more likely to reflect the degree of antigenic relatedness between different HCV variants than evolutionary distances between nucleotide sequences. A similar set of distinct approximately normally distributed values was observed for type, subtype and isolates comparisons (Table 1*b*), each showing numerically distinct median values and non-overlapping 95th percentile ranges. The amino acid sequence differences between major genotypes (38%; range 25–50%) are consistent with antigenic differences greater than those found between subtypes and isolates (median values 17% and 4%), although this analysis cannot compute the differing contributions to changes in antigenicity produced by biochemically similar or dissimilar amino acid substitutions (see below).

Description of NS-4 amino acid sequences for HCV types 4 to 6

The inferred amino acid sequence of 111 residues translated from the NS-4a sequences between positions 1653–1764 (Choo *et al.*, 1991) includes the region corresponding to the immunoreactive clone 5-1-1 (1683–1735) (Fig. 3). In the region compared, only 47 sites are conserved between all variants analysed, while many of the variable sites contain substitutions of amino acids possessing side-chains with different biochemical properties, and which could potentially alter the structure and antigenic properties of the protein (highlighted in bold). Previous epitope mapping of this region for genotypes 1a, 1b, 2b, and 3a (Simmonds *et al.*, 1993*c*) identified regions of greatest antigenicity at positions 1691–1708

			-245	-235	-225	-175	-165	-155	-145
1a	1	PT-1	TGAGTGTCGT	GC	GGACCCCCCC	ACCGGAATTG	CCAGGACGAC	CGGGTCCTTT	C--TTGGAT-CAA
1b	8	J	.....T..	..	.....	.....	.....	.....	.--.....-...
	34	HCVUK	.....	..	.....	.....	.....	.....	.--.....-...
1c	35	4TY4	.....	A.	.....	.....C.	.....	.....	.--.....-A..
1d	16	HC-G9	.....	..	.....	.....	.....	.....	.--.....-T..
2a	17	J6	.....	A.	..C.....	.....	..G...A...	.....	.--.....-A..
	20	T351	.....	A.	..C.....	.....	..G...A...	.....	.--.....-A..
2b	23	J8	.....	A.	..C.....	.....	..G.A.A...	.....	.--.....-A..
2c	36	T983	.....	A.	..C.....	.....	..G...A...	.....	.--.....-A..
3a	33	T1787	C.....	..	.....	.....C.	.TG..GT...	.....	.--.....A-...
3b	37	B 4	C.....	..	..C.....	.....C.	..G...T...	.....	.--.....A-...
4a	39	EG-1	.....T..	A.	.....	.....C.	..G...T...	.....	.--.....-T..
	40	EG-13	.....T..	..	.....	.....C.	..G...T...	.....	.--.....-T..
	41	EG-21	.....T..	..	.....	.....C.	..G...T...	.....	.--.....-T..
	42	EG-24	.....T..	A.	.....	.....C.	..G...T...	.....	.--.....-T..
	43	EG-25	.....T..	A.	.....	.....C.	..G...T...	.....	.--.....-T..
	44	EG-33	.....T..	A.	....T.....	.....C.	..G...T...	.....	.--.....-T..
5a	45	T478	.....	A.	.....	.....	..G...T...	.....	.--.....-T..
	46	SC6	.....A	A.	.....	.....	..G...T...	.....	.--.....-A..
	47	SC23	.....A	A.	.....	.....	..G...T...	.....	.--.....-A..
	48	SC24	.....	A.	.....	.....	..G...T...	.....	.--.....-...
6a	49	HK-4	NNNNNNNNN	NN	.....	.....	.....	.....	.CA.....CA..
	0	T3950	.....	A.	.....	.....	.....T...	.....	.CA.....CA..

			-135	-125	-115	-105	-95	-85	-75
1a	1	PT-1	CCCGCTCAAT	GCCTGGAGAT	TTGGGCGTGC	CCCCGCAAGA	CTGCTAGCCG	AGTAGTGTTG	GGTCGCG
1b	8	J	.....	.....	.....	.....G...	.....	.....	.....
	34	HCVUK	.....	.....	.....	.....	.....	.....	.....
1c	35	4TY4	.....	.....A..	.....	.....	.....	.....	.....
1d	16	HC-G9	.....	.....	.....	.....	.....	.....	.....
2a	17	J6	...A...T..	...C..TC..	.....	.....	.....	.....C....	...T...
	20	T351	...A...T..	...C..CC..	.....	.....	.....	.....C....	...T...
2b	23	J8	...A...T..	.T.C..TC..	.....AC..	.....	.....	.....C....	...T...
2c	36	T983	...A...T..	...C..CC..	.....	.....	.....	.....	...T...
3a	33	T1787	.....	A..CA..A..	.....	.....G...	TCA.....	.....	.....
3b	37	B 4	.....	...C...A..	.....	.....G...	TCA.....	.....	.....
4a	39	EG-1	.....	...C...A..	.....	.....G...	.....	.....	.....
	40	EG-13	.....	...C...A..	.....	.....G...	.....	.....	.....
	41	EG-21	.....	...C...A..	.....	.....G...	.....	.....	.....
	42	EG-24	.....	...C...A..	.....	.....G...	.....	.....	.....
	43	EG-25	.....	...C...A..	.....	.....G...	.....	.....	.....
	44	EG-33	.....	...C...A..	.....	.....G...	.....	.....	.....
5a	45	T478	.....	...C.....	.....	.....G...	.....	.....	.....
	46	SC6	.....	...C.....	.....	.....G...	..A.....	.....	.....
	47	SC23	.....	...C.....	.....	.....G...	.....N	NNNNNNNNN	NNNNNNN
	48	SC24	.....	...C.....	.....	.....G...	.....	.....	.....
6a	49	HK-4	.....	.....	.....	.....	.....	.....	.....
	50	T3950	.....	.....	.....	.....	.....	.....C....	...T...

Fig. 1. Comparison of sequences in the 5'NCR of different genotypes of HCV from variants used for sequence determination in NS-4 (listed in the legend to Fig. 2); differences from the HCV prototype strain HCV-PT (Choo *et al.*, 1991) are indicated. The genotype assigned to each sequence, following proposed nomenclature (Simmonds *et al.*, 1994*a*) on the basis of the sequence in the 5'NCR is shown in the first column. Nucleotide sequences −232 to −225, −214 to −168 and −153 to 145 are omitted as they are invariant between the sequences shown. (.), Sequence identity to HCV-PT; (−), gap introduced into the sequence to preserve alignment; (N), sequence not determined.

(designated region 1) and 1710–1728 (region 2), underlined in the prototype sequence.

Genotypes 3a, 3b, 4a, 5a, and 6a show similar amino acid sequences in antigenic region 1, although the type 4a sequences contain a possible antigenically significant change to a glutamine residue at position 1691 in place of basic amino acids (Fig. 3). Type 3b has a glutamine in place of an otherwise universal glutamate at position

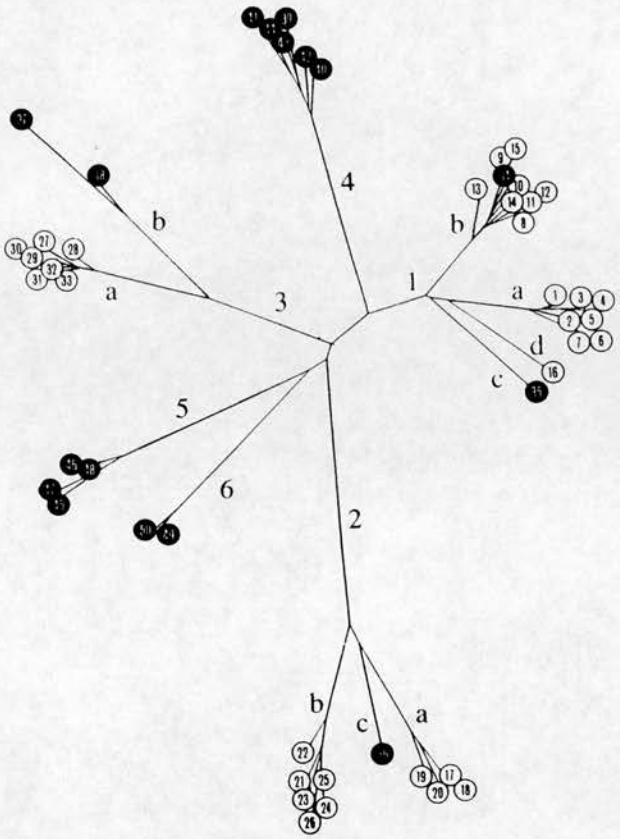

Fig. 2. Phylogenetic analysis of a 342 base region of NS-4 (nucleotide positions 4941–5282) of sequences from HCV genotypes 1 to 6. 1–33, previously published sequences; 34–50, new NS-4 sequences obtained in this study corresponding to more recently described genotypes. Phylogenetic groups are labelled according to proposed nomenclature; sequence 13 (labelled 1d) from Indonesia is described as '1c' in Okamoto *et al.* (1994) and '1d' in Hotta *et al.* (1994). Type 4 sequences from Egyptian blood donors correspond to variants described as '4a' in Simmonds *et al.* (1993*a*), but are different from '4a' in Bukh *et al.* (1993). Sources of published NS-4A sequences (number, clone name, geographical origin, genotype, source reference) are as follows: (1) PT-1, USA, 1a (Choo *et al.*, 1991); (2) HCV-H, USA, 1a (Inchauspe *et al.*, 1991); (3–7) T16, T42, T77, T1801, T1825, Scotland, UK, 1a (Simmonds *et al.*, 1993*c*); (8) HCV-J, Japan, 1b (Kato *et al.*, 1990); (9) HCV-BK, Japan, 1b (Takamizawa *et al.*, 1991); (10) HPCJTA, Japan, 1b (Tanaka *et al.*, 1992); (11, 12) J483, J491, Japan, 1b (Okamoto *et al.*, 1992*a*); (13) HCVJKIG, 1b (M. Honda and others, GenBank number X61596); (14) T, Taiwan, 1b (Chen *et al.*, 1992); (15) HPCGENOM, China, 1b (X. Bi and others, GenBank number L02836); (16) HC-J9, Indonesia, 1d (Okamoto *et al.*, 1994); (17) HC-J6, Japan, 2a (Okamoto *et al.*, 1991); (18) K2A, Japan, 2a (Enomoto *et al.*, 1990); (19) D11353, Japan, 2a (Sarashina *et al.*, 1993); (20) T351, Scotland, UK, 2a (Simmonds *et al.*, 1993*c*); (21) Clone A, Clone E, Japan, 2b (Tsukiyama Kohara *et al.*, 1991); (23) HC-J8, Japan, 2b (Okamoto *et al.*, 1992*b*); (24–26) T59 (E-b12), T810, T940, Scotland, UK, 2b (Simmonds *et al.*, 1993*c*); (27, 28) BR36-20, HD10-1, Brazil, 3a (Stuyver *et al.*, 1993*b*); (29–33) T26 (E-b3), T32 (E-b7), T38 (E-b2), T40 (E-b1), T1787, Scotland, UK, 3a (Simmonds *et al.*, 1993*c*). Sources of the new NS-4A sequences are as follows: (34) HCVUK, UK, 1b (Garson *et al.*, 1990); (35) 4TY4, Lebanon, 1c (Simmonds *et al.*, 1993*a*); (36) T983, Scotland, UK, 2c (Simmonds *et al.*, 1993*a*); (37) B4, Bangladesh, 3b; (38) B9, Bangladesh, 3b; (39–44) EG-1, EG-13, EG-21, EG-24, EG-25, EG-33, Egypt, 4a (Simmonds *et al.*, 1993*b*); (45) T478, UK, 5a; (46–48) SC6, SC23, SC24, South Africa, 5a (Davidson *et al.*, 1994); (49, 50) HK-4, T3950, Hong Kong, 6a (Simmonds *et al.*, 1993*a*).

1699 and, along with 3a, a tyrosine residue instead of phenylalanine at 1705. Region 2 displays a high degree of antigenically significant type-specific variation between all the genotypes. The type 5a sequences contain a glutamate-alanine-arginine motif (EAR) at position 1720–1722, while type 6 has a specific motif alanine-glutamate-residue x-glutamine-glutamine (AE-QQ) at 1719–1723. Genotype 4a contains four amino acid changes of biochemical significance in this region, i.e. lysine at position 1713, leucine at 1717, histidine at 1720 and glutamine at 1724. A lesser degree of sequence variability is found amongst sub-types of HCV. The single examples of types 1c and 1d possess specific histidine residues at positions 1720 and 1723 respectively, while type 2c has threonine, histidine and arginine residues at positions 1715, 1722 and 1724. Sub-type 3b contains specific amino acid residues at positions 1720 (proline) and 1726 (glutamine).

Development of an ELISA to detect genotype-specific antibody

Peptides corresponding to the two previously identified antigenic regions of NS-4 (underlined in Fig. 3) for genotypes 1 to 6 were synthesized in branched form and used as antigens for detection of genotype-specific antibody. Consensus sequences for regions 1 and 2 were decided upon for each genotype, although it was clear from the outset that the region 1 peptides for genotypes 4 to 6 were all very similar to 3a (Fig. 4). In some cases, it was necessary to synthesize more than one peptide for a particular genotype to cover intra-typic sequence heterogeneity, but not to introduce combinations of amino acid polymorphisms that did not exist in native sequences. For example, different consensus sequence peptides were synthesized for each of the three subtypes of type 2 (Fig. 4).

The format of the current assay differs considerably from those of previously described serotyping assays that were based upon the coating of different antigens corresponding to different genotypes into different wells (Tsukiyama Kohara *et al.*, 1993; Yunomura *et al.*, 1994; Tanaka *et al.*, 1994; Mondelli *et al.*, 1994). In the current assay, all wells were coated with peptides from all six genotypes. Type-specific antibody was detected by competition between the solid phase antigens with a 100-fold molar excess of peptides added in solution with the plasma or serum sample. For example, detection of genotype-specific antibody to type 1 is achieved by

Table 1. *Distribution of pairwise nucleotide (a) and amino acid (b) sequence distances between HCV types, subtypes and isolates*

		Type	Subtype	Isolate
	No. of pairwise comparisons...	959	145	121
(a) Nucleotide distances				
Median		0·617	0·275	0·083
Range	Min	0·359	0·180	0·000
	Max	0·888	0·380	0·146
95% percentile	Lower	0·449	0·228	0·006
	Upper	0·815	0·364	0·135
Mean		0·617	0·279	0·079
SD		±0·113	±0·030	±0·031
(b) Amino acid divergence				
Median		0·381	0·168	0·037
Range	Min	0·230	0·106	0·000
	Max	0·550	0·240	0·115
95% percentile	Lower	0·245	0·108	0·000
	Upper	0·502	0·221	0·094
Mean		0·374	0·164	0·041
SD		±0·071	±0·029	±0·023

addition of peptides from genotypes 2 to 6 to the test serum or plasma samples (see Methods). This method guarantees that only those antibodies that can bind to the solid phase are those that do not cross-react with heterologous genotypes to HCV.

The frequency of antibody reactivity to type-specific antibody to this region of NS-4 was assayed using a total of 210 PCR-positive plasma samples from blood donors or patients with chronic hepatitis, and whose circulating genotype was determined by RFLP as recently described (Davidson *et al.*, 1995). To investigate the frequency of non-specific reactivity to the NS-4 peptides, we also tested a total of 20 anti-HCV negative blood donor control sera. For each sample, we recorded the absorbance produced upon reaction with all of the peptides coated onto the solid phase (unblocked control), in competition with all peptides in solution (blocked control), and finally a series of six wells for detection of type-specific antibody to each of the genotypes individually (Fig. 5).

The relative proportion of type-specific and cross-reactive antibody to the epitopes presented by the NS-4 peptides was estimated by comparison of the median and interquartile ranges of the absorbance values upon reaction with homologous NS-4 peptides with the unblocked control (Fig. 5). The absorbance value from the blocked control was in each case subtracted from the values obtained from the other wells to control for any non-specific binding to the solid phase. For all genotypes, antibody reactivity was almost always confined to peptides of the same genotype as determined by PCR–RFLP typing, whereas binding was not observed amongst any of the anti-HCV negative controls (Fig. 5A). For the anti-HCV positive sera, a wide range of antibody reactivity was observed, both against the homologous type peptide and in the unblocked control well. For each genotype, the median values were greater in the unblocked wells than they were to the homologous peptide, indicating a mixture of antibody to type-specific and cross-reactive epitopes. Precise measurement of the relative proportions could not be carried out without titration of the samples, because there was not a linear relationship between absorbance and amount of antibody for absorbance values greater than 1 (unpublished observations), and because absorbance values recorded for many of the samples were greater than 2·0 (see Methods).

For genotypes 4, 5 and 6, levels of type-specific antibody were markedly lower than for types 1 to 3, while total anti-NS-4 antibody (unblocked control) was similar to that found in the other genotypes. These findings could be explained by the greater similarity of the first antigenic region of genotypes 4, 5 and 6; it is possible that the principal component of the type-specific reactivity is directed solely to the more divergent second region. Little difference was found in total reactivity to NS-4 or to homologous peptides between subtypes 1a and 1b, or between 2a and 2b, apart from the slightly weaker reactivity of type 1b antibody to the type 1 peptides (Fig. 5C). As described above, this could be the consequence of a greater similarity of type 1b NS-4 sequences to other genotypes than type 1a. For example, the alanine (A), serine (S) and glutamine (Q) substitutions at positions 1711, 1712 and 1722 (all in region 2) found in the 1b sequences are shared with type 2 (Fig. 3), and antibody reactivity with epitopes dependent upon these amino acid residues would be more likely to be absorbed by heterologous peptides.

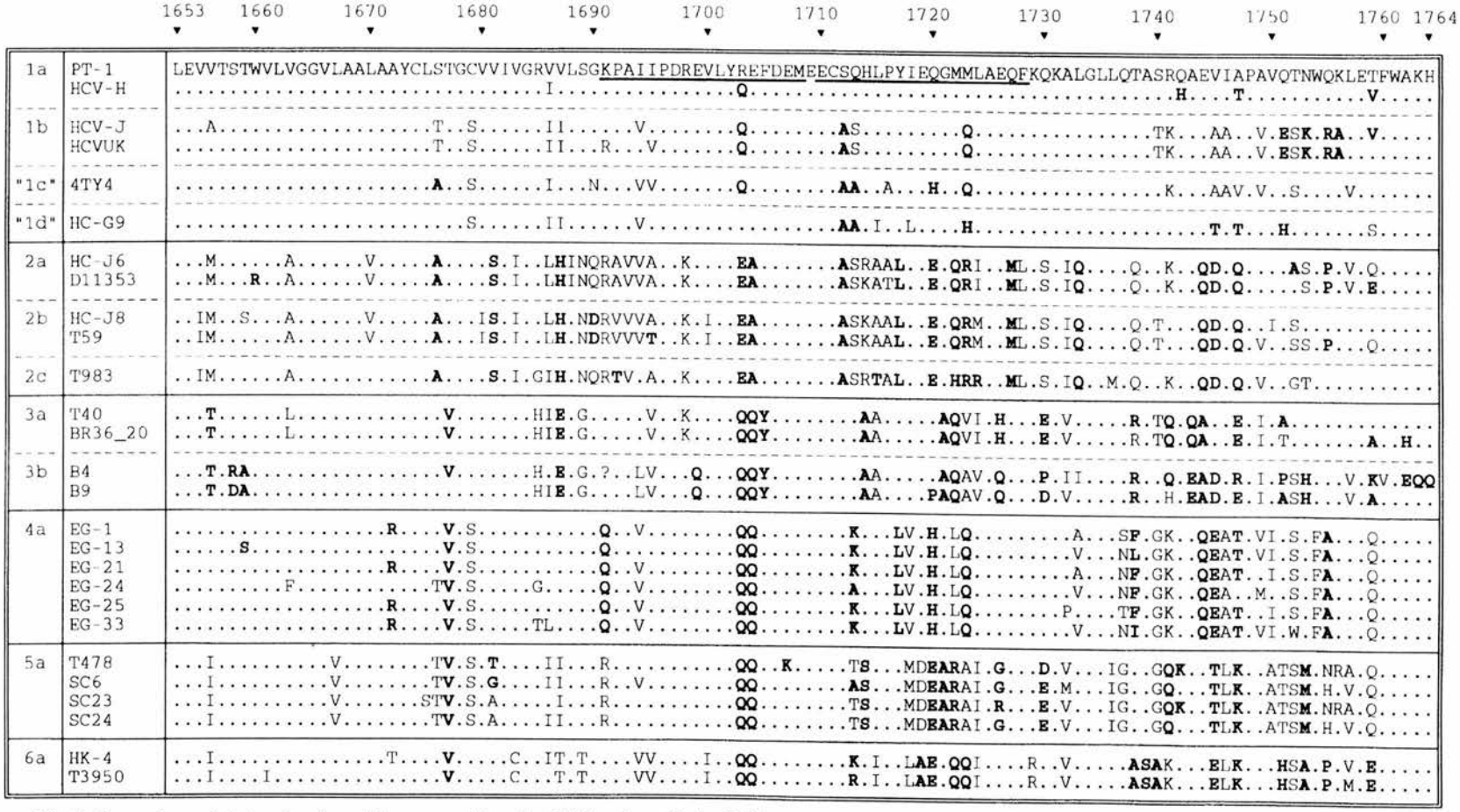

Fig. 3. Comparison of deduced amino acid sequences from the NS-4 region with the HCV prototype sequence (HCV-PT; Choo *et al.*, 1991). Antigenic regions identified by previous epitope mapping are underlined (Simmonds *et al.*, 1993*c*). Substitutions of biochemically distinct amino acid residues that could affect the antigenicity of the encoded protein are highlighted in bold. (.), Sequence identity to HCV-PT; (–), gap introduced into the sequence to preserve alignment; (?), sequence not determined.

TYPE	REGION 1	REGION 2
1	KPAIVPDREVLYREFDEM R..VI.....Q.....	ECSQHLPYIEQGMMLAEQF .....A.....
2a 2b 2c	RAV.A..K....EA.... RVVVT..K.I..EA.... RTV.A..K....EA....	..ASRAAL..E.QRI..ML ..ASRAAL..E.QRI..ML ..ASRTAL..E.HRI..ML
3	...L...K....QQY...	....AA.....AQVI.H..
4	Q..VI.....QQ....	...K...LV.H.LQ.....
5	R...I.....QQ....	...TS...MDEARAI.G..
6	...VV....I..QQ....	...R.I..LAE.QQI....

Fig. 4. Sequences of peptides used for ELISA, corresponding to antigenic regions 1 (positions 1691–1708) and 2 (1710–1728) of HCV genotypes 1 to 6. For types 1 and 2, more than one peptide was used to allow for sequence heterogeneity within the genotype.

The presence of type-specific antibody within an individual sample was scored by comparison of the antibody reactivity within each well with that of the blocked control. Samples with absorbance values two or more times that of the blocked control were considered positive, provided the absorbance was greater than 0·1. Using this scoring system, we compared the detection of type-specific antibody by ELISA with the circulating genotype detected by PCR (Table 2). We found an overall concordance of 97% (177 out of 182 samples reactive in the ELISA contained type-specific antibody to the genotype detected by PCR), with no significant difference between genotypes. Only single instances were found where samples contained antibody to a different genotype in the RFLP assay. No sample showed antibody to more than one genotype. The frequency of detection of type-specific antibody was 87%, although variation was observed between genotypes. High rates (92–100%) were found for genotypes 1a, 2a, 2b and 3a, while type 1b and 4 were lower at 83% and 81% respectively. Detection rates for types 5 and 6 were based upon relatively fewer samples (16 and 6), and require further investigation with more extensive panels of genotyped samples.

Discussion

Sequence variability in the NS-4 region

Variability in the NS-4a region of the HCV genome between positions 4941–5282 appeared comparable to relationships between genotypes and subtypes of HCV in other coding regions of the genome (Simmonds *et al.*, 1993*a*; Bukh *et al.*, 1993, 1994). In each case, three levels of sequence variability were found, with a clear distinction into major genotypes and subtypes that has been proposed as the basis for classification of HCV (Simmonds *et al.*, 1994*a*). The region of NS-4a analysed in this report shows similar degrees of sequence divergence to the other non-structural proteins of HCV, NS-3, NS-4B, NS5A and -5B. It is more conserved than E1 with nucleotide evolutionary distances ranging from 0·46–0·80 between major genotypes, compared with 0·43–0·85 for E1 (positions 574–1149, Bukh *et al.*, 1993), and much more than core (0·13–0·25, Chan *et al.*, 1992, and our unpublished observations). Similar relationships were found between the inferred amino acid sequences of the different parts of the genome (data not shown).

Pairwise comparisons of amino acid sequences of major genotypes showed 23–55% variation, and did not significantly overlap with any of those between subtypes (11–24%) nor between individual variants within a subtype (0–12%). Furthermore, genotype identifications of samples on the basis of sequence data obtained elsewhere in the genome, such as NS-5 (Fig. 2, seq. nos 6, 7, 20, 24, 25, 29–33) or the 5′NCR (Fig. 1; Fig. 2, seq. nos 34–50) are in each case consistent with the groupings observed in the NS-4 tree (Fig. 2), providing further evidence that sequence variability in NS-4a is representative of the virus as a whole.

Frequent amino acid substitutions were found between genotypes in regions of NS-4a previously shown to be antigenic by epitope mapping (Simmonds *et al.*, 1993*c*), apart from the unexpected similarity found within the first antigenic region for genotypes 4, 5 and 6 (Figs 4 and 5). It is possible that this accounts for the slightly reduced sensitivity of the ELISA for type-specific antibody to these genotypes, and suggests the use of peptides from other regions of NS-4 as additional antigens for the ELISA. For example, we have been able to detect type-specific antibody using peptides from part of NS-4 downstream from the 2nd antigenic region that is highly variable between genotypes (unpublished data), and are currently investigating the possibility of using this region in a modified and more sensitive serotyping ELISA.

Detection of genotype-specific antibody

The incubation of samples with blocking concentrations of heterologous peptides provides an effective and sensitive assay for genotype-specific antibody. The method largely eliminates the possibility of false detection of antibody through shared epitopes between different genotypes, a problem that is inevitable and limiting for assays based upon indirect ELISAs (Tanaka *et al.*, 1994; Tsukiyama Kohara *et al.*, 1993; Yunomura *et al.*, 1994; Mondelli *et al.*, 1994). In contrast, the detection of reactivity to more than one genotype in the assay described in this report is more likely to indicate antibody produced by multiple infection with different HCV variants. This is shown by the previous finding of

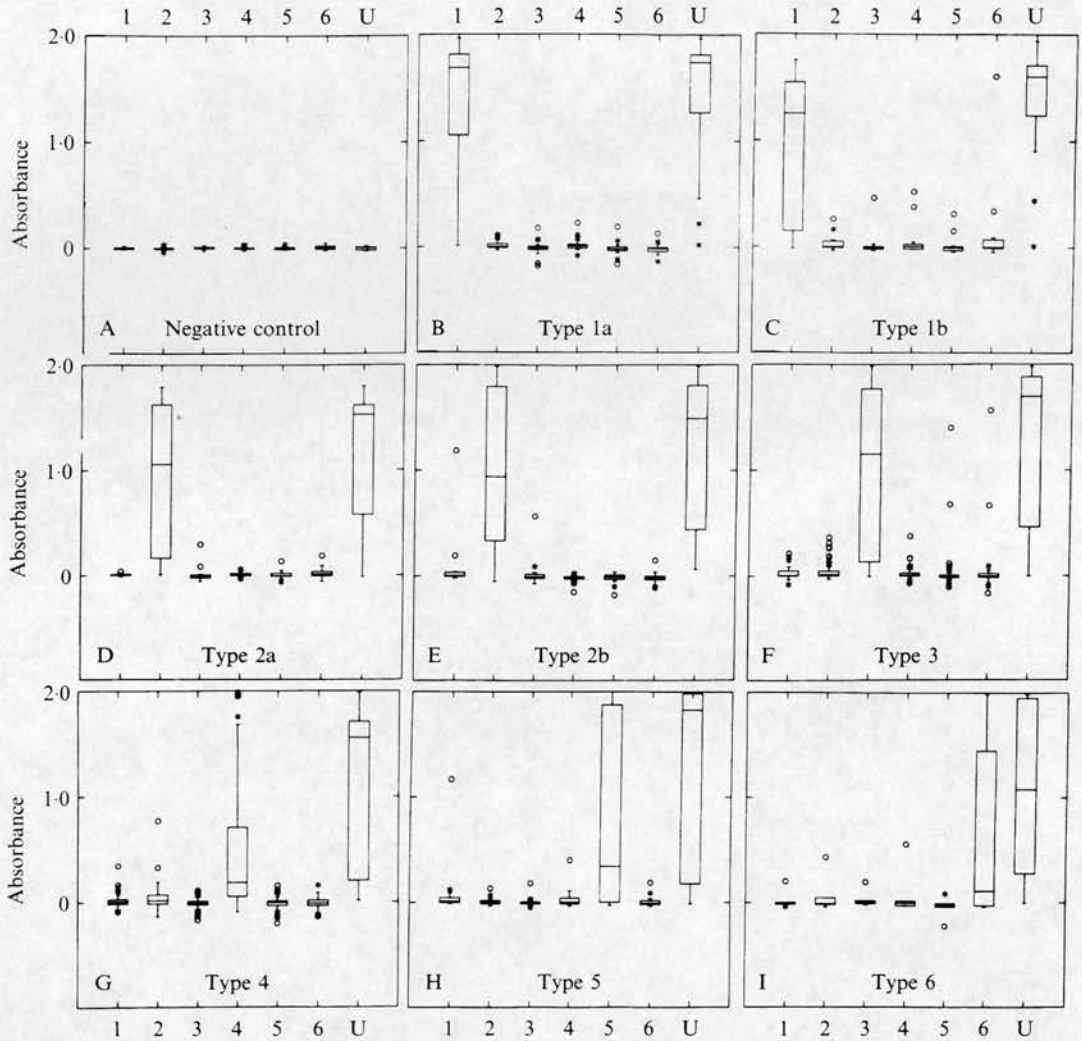

Fig. 5. Box plot of the net absorbance value, *A* (*A* − blocked control) for negative controls (A, $n = 20$), and samples representing each HCV genotype [1a − B ($n = 24$), 1b − C ($n = 9$), 2a − D ($n = 13$), 2b − E ($n = 17$), 3 − F ($n = 50$), 4 − G ($n = 48$), 5 − H ($n = 10$), 6 − I ($n = 6$)] against type-specific NS-4 epitopes from each genotype (wells 1–8) and to the unblocked control (well 7). Median value and interquartile ranges are indicated by the internal line and the upper and lower sides of the box; the single line represents values lying within 1·5 times the interquartile range. Outliers with values greater than 1·5 or 3 times the interquartile range are indicated by (*) and (○) symbols respectively (see Methods).

a high frequency of antibody to genotypes 1 and 2, or 1 and 3 in haemophiliacs exposed to multiple contaminated batches of clotting factor (Jarvis *et al.*, 1994), which contrasts with the reactivity to single genotypes found in this study for all 182 reactive samples from blood donors and hepatitis C patients.

Few of the amino acid differences between HCV subtypes, for example between 1a and 1b, would be expected to lead to significant antigenic variability. We have previously found that reactivity to peptides derived from type 1a and 1b sequences produced almost identical patterns of reactivity upon epitope-mapping the NS-4a region, (Simmonds *et al.*, 1993*c*). Similarly, sera from type 2a- and 2b-infected blood donors showed no consistent differences in reactivity with overlapping peptides from type 2b. This antigenic similarity (particularly between 1a and 1b) has provided an obstacle for the development of an ELISA to detect infection with different subtypes for all but a small proportion of samples. It remains possible that other (more variable) regions of the genome might be more suitable for such an assay. Alternatively, the sequence variability that does exist between subtypes might lead to antigenic differences between native proteins absent from linear peptides. However, the apparent inability of an NS-4 based serotyping assay based upon recombinant polypeptides to separately identify antibody to subtypes (Tanaka *et al.*, 1994) argues against the second possibility. Fur-

Table 2. *Detection of type-specific antibody in plasma samples of HCV-infected patients and blood donors of known genotype*

Genotype*		Serotype†									Sensitivity (%)‡	Concordance (%)§
		1	2	3	4	5	6	NTS	NR	Total		
1 ‖	All	**35**	–	–	–	–	1	1	2	39	92·3	97·2
	a	***24***	–	–	–	–	–	*0*	*1*	*25*	*96·0*	*100*
	b	***9***	–	–	–	–	*1*	*1*	*1*	*12*	*83·3*	*90·0*
2 ‖	All	1	**30**	–	–	–	–	0	1	32	96·9	96·8
	a	–	***13***	–	–	–	–	*0*	*1*	*14*	*92·9*	*100*
	b	*1*	***17***	–	–	–	–	*0*	*0*	*18*	*100*	*94·4*
3		–	1	**50**	–	–	–	2	2	55	92·7	98·0
4		–	1	–	**49**	–	–	9	3	62	80·6	98·0
5		1	–	–	–	**10**	–	2	3	16	68·8	90·9
6		–	–	–	–	–	**4**	1	1	6	66·6	100
Total		37	32	50	49	10	5	15	12	210	87·1	97·3
Neg.		–	–	–	–	–	–	–	20	20	–	–

* Genotype determined by RFLP of 5′NCR sequences as previously described (McOmish *et al.*, 1994; Davidson *et al.*, 1994).

† Serotype: detection of type-specific antibody to HCV genotypes 1–6 in these samples; no sample showed type-specific antibody to more than one genotype. Results concordant with genotyping by RFLP shown in bold. NR, non-reactive; NTS, non-type-specific antibody reactivity to NS-4 peptides.

‡ Number typeable by ELISA/number tested.

§ Number concordant with genotype determination by RFLP/number typeable by ELISA.

‖ Totals for type 1 and 2 samples; results for subtypes 1a, 1b and 2a, 2b are shown in italics.

thermore, there is little evidence that antigens derived from other regions of the HCV genome might be more appropriate for the detection of antibody to subtypes. For example, the degree of amino acid sequence variability in the E1 gene is similar to that found in NS-4 (see above), while E2 contains antigenic regions that are so variable that they are not conserved within a subtype.

The frequent detection of specific antibody to the circulating genotype identified by PCR indicates a potential role for this serotyping assay as an alternative to conventional PCR-based typing assays. However, the assay is dependent upon a normal immune response to virus infection, and would not necessarily be able to detect NS-4a antibody in individuals who are severely immunosuppressed, or in samples collected around the time of seroconversion following primary infection. In individuals who are multiply-exposed, antibody to more than one genotype was frequently found, even though only one circulating genotype was detected by PCR (Jarvis *et al.*, 1994), indicating that the ELISA may detect past as well as current infection. The appearance of new type-specific antibody in those haemophiliacs in whom the circulating genotype spontaneously changed was frequently delayed by several months, providing a further cause of discrepant results in this risk group (Jarvis *et al.*, 1994).

Information on risk behaviour and previous exposure to HCV was not available for the blood donor and hepatitis C patients in this study, and it is possible that past infection with different genotypes accounted for the discrepant results in Table 2. It is also possible that the peptides used do not represent the full range of antigenic variation within each genotype; to take one example from Table 2, epitopes present on an unusual variant of type 1 might also be found in type 6 NS-4a sequences. This possibility could be explored by sequence analysis of the NS-4a region sequences of samples producing such discrepant results. It would also exclude the possibility that the differences between genotyping in the 5′NCR by PCR and serotyping by ELISA result from infection with recombinants of HCV, with sequences corresponding to different genotypes in different parts of the genome. However, sequence analysis of variants of HCV in more than one region of the genome has so far failed to produce any evidence for the existence of recombinant viruses *in vivo* (Figs 1 and 2) (Simmonds *et al.*, 1994*b*; Lau *et al.*, 1994), and must be considered an unlikely explanation for the discrepant results reported here.

Clinical utility of genotyping

Although there is no evidence for genotypes of HCV that are completely non-pathogenic or perhaps non-hepatotropic, there are many reports of differences between them in the rate of disease progression, and particularly in the probability of achieving a sustained response to anti-viral treatment (reviewed in Dusheiko, 1994). These clinical differences indicate the potential utility for identification of the infecting genotype in patients for

patient selection, and in calculating the most effective duration and dose of interferon treatment to achieve a long-term response. The serological typing method described here may be of major value in service laboratories that require a quick and simple assay for identification of HCV genotypes, without the necessity to purchase expensive items of equipment for PCR or to introduce unfamiliar working techniques into the laboratory. The use of different combinations of competing peptides in the ELISA allows the design of different formats of the assay to maximize its clinical utility and ease of use. For example, in Europe it may be more practical to screen patients for genotypes 1, 2 and 3 separately, and to detect other genotypes (4, 5 and 6) together by competition with peptides from genotypes 1, 2 and 3 only. It would also be possible to incorporate additional peptides corresponding to newly discovered major genotypes (e.g. Apichartpiyakul *et al.*, 1994) into the ELISA for screening populations where these variants occur.

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Response to interferon-α of Egyptian patients infected with hepatitis C virus genotype 4

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SUMMARY. Hepatitis C virus (HCV) genotype 4 is the principal HCV genotype found in Egypt and the Middle East. Little is known concerning its propensity to cause disease and the frequency with which infected individuals respond to interferon-α (IFN-α). We have investigated the response to treatment in a cohort of 100 chronic hepatitis C patients infected with genotype 4. All patients had biopsy-proven chronic active liver disease. Each was treated with 3 million units (MU) IFN-α, thrice weekly. Response was monitored, in 92 patients who completed treatment, by alanine aminotransferase (ALT) measurements and by polymerase chain reaction (PCR) for HCV. ALT levels remained abnormal in 64 patients during treatment (69.6%). Of the 28 patients who showed a biochemical response during treatment (30.4%), 18 maintained this over the 6-month post-treatment period. Amongst the sustained biochemical responders, HCV RNA was cleared from serum in only four of the 18 (22.2%) in this period. Histological improvement was observed in 26/51 (50.9%) of the patients who had a second biopsy.

Hence, patients infected with HCV genotype 4 show a poor response to IFN-α therapy compared with genotypes 2 and 3, but a similar response to IFN-α compared with those infected with type 1b HCV. These findings have major implications for treatment strategies in the Middle East, including Egypt, where HCV genotype 4 is widely distributed.

Keywords: antiviral, Egypt, genotype 4, interferon treatment.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus that has been identified as the principal cause of post-transfusion non-A, non-B hepatitis [1,2]. HCV shows substantial sequence diversity, and sequence comparisons in E1 and non-structural regions of the genome has led to classification of HCV into a series of genotypes that show distinct geographical distributions [3,4]. Currently HCV can be classified into six major genotypes that typically differ from each other by up to 30–35% in nucleotide sequence in non-structural proteins, and between 35–50% in the E1 and E2 regions encoding putative envelope glycoproteins. The 5' non-coding region (5'NCR) is highly conserved and genotyping assays are frequently based upon this region, where specific nucleotide differences between HCV genotypes can be detected by cleavage of amplified DNA sequences by restriction endonucleases (restriction fragment length polymorphism, RFLP) [5], or by hybridization to genotype-specific probes [6,7].

In the Middle East, including Egypt, HCV type 4 is the most frequently detected genotype in blood donors and patients with hepatitis C [8–10]. A recent study on HCV genotyping in Egyptian patients showed a prevalence of type 4 in 92% of HCV antibody (HCVAb) positive patients (unpublished data). HCV genotypes may differ in their biological properties, such as severity of disease and response to interferon. For instance, individuals infected with types 2 and 3 appear to respond

Abbreviations: ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN-α, interferon-α; 5'NCR, 5' non-coding region; RFLP, restriction fragment length polymorphism.

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more frequently to interferon-α (IFN-α) therapy than do those with type 1 [11,12], although little is currently known concerning the response of genotypes 4, 5 and 6. In this study, we have studied responses to treatment in a large group of HCV-infected patients from the Cairo Liver Centre who were infected with type 4 HCV. Responses were assessed by measurement of alanine aminotransferase (ALT) and clearance of viraemia by polymerase chain reaction (PCR), and are compared with those previously reported for other HCV genotypes.

MATERIALS AND METHODS

Study subjects

One hundred patients were identified as HCVAb positive by second generation enzyme immunoassay (EIA-2), and were viraemic as assessed by PCR. The study group who completed treatment comprised 77 males and 15 females, with a mean age of 39.5 years. Each patient was treated with IFN-α2a (Roche) at a dose of 3 million units (MU) thrice weekly (t.i.w.) rising occasionally to 5 MU t.i.w., for a total duration of 6 months. Eight patients were withdrawn from the study owing to intolerable side effects, allowing complete evaluation in 92 patients. Risk factors and clinical presentation were evaluated in these patients.

Monitoring of response to treatment

Liver biopsy was performed on 85 patients before IFN-α treatment and on 51 patients 6 months after the end of therapy. Patients' refusal prohibited liver biopsy in the rest of the study group. ALT levels were monitored monthly during the course of therapy in all patients, and for the first 6 months following treatment in those showing an initial response. Responders to IFN therapy were categorized as:

1 non-responders (NR), maintenance of abnormal ALT level during treatment;
2 complete responders (CR), steady normalization of ALT level by the end of 6 months of treatment;
3 sustained responders (SR), complete response lasting for 6 months after discontinuation of therapy.

PCR and genotyping

HCV RNA sequences were amplified from plasma by nested PCR using primers from the highly conserved 5'NCR as previously described [13,14]. HCV genotypes were determined by RFLP analysis of amplified DNA using *Hae*III/*Rsa*I and *Mva*I/*Hin*fI as previously described [15].

RESULTS

Clinical presentation

The main risk factor for HCV infection in this study group was a past history of intravenous antischistosomal therapy (30.4%; Table 1). Other potential exposures to HCV included past blood transfusion (15.2%), previous surgery (15.2%) and dental therapy (21.7%). Over 70% of the patients were asymptomatic and HCV infection was discovered incidentally during a medical examination before employment or surgery. About 20% of patients suffered from flatulent dyspepsia, possibly as a result of congestive gastropathy secondary to portal hypertension. Only 10% of patients presented with fatigue.

Histopathological and virological findings

The predominant pathological findings among the study patients were chronic active hepatitis (CAH) +

Table 1 Pre-treatment characteristics of the study group

Total *n*	92
M:F	77:15
Mean age (range)	39.5 (32–50) years
Mean ALT	123 IU ml^{-1}
Mean platelet count	143 000 mm^{-3}
	n (%)
Clinical presentation	
Asymptomatic	64 (70)
Flatulent dyspepsia	18 (20)
Fatigue	10 (10)
Risk factor	
Antischistosomal parenteral therapy	28 (30.4)
Dental treatment	20 (21.7)
Previous surgery	14 (15.2)
Blood transfusion	14 (15.2)
More than one risk factor	16 (17.4)
Biopsy appearance	
Chronic lobular hepatitis	8 (9.4)
Chronic persistent hepatitis	16 (18.8)
Chronic active hepatitis	22 (25.9)
Chronic active hepatitis + cirrhosis	39 (45.9)

cirrhosis (45.9%) and CAH alone (25.9%) (Table 1). Chronic persistent hepatitis was found in 18.8% and chronic lobular hepatitis in 9.4%. All patients were infected with genotype 4 HCV as identified by RFLP analysis of amplified sequences from the 5'NCR using restriction enzymes *Hae*III/*Rsa*I and *Mva*I/*Hin*fI.

Response to IFN-α treatment

A total of 28 patients (31%) showed a complete response to IFN-α treatment as monitored by normalization of ALT (Table 2). Of these, 18 maintained normal ALT levels over the 6 months following treatment, while the remainder relapsed. All patients were HCV RNA positive before the start of IFN-α treatment. PCR was repeated 6 months after the end of therapy to calculate the sustained virological response to treatment. Serum HCV RNA was negative in only four of the 18 individuals (22%) showing a sustained normalization of ALT. Long-term sustained virus clearance was thus obtained only in four of the 92 members of the study group (4%). Histological improvement by histology activity index (HAI) score was observed in 26 of 51 (50.9%) patients in whom a post-treatment liver biopsy was carried out. Biopsies in those who responded to treatment showed evidence of an anti-inflammatory response with a decrease in periportal and lobular inflammation (Table 3). Patients with cirrhosis were less likely to respond to treatment than those without evidence of fibrosis.

DISCUSSION

A large number of published and ongoing investigations have shown differences between HCV genotypes in the frequency of response to interferon therapy. However, almost all of these have been confined to the investigation of an extremely restricted range of genotypes. This particularly applies to studies reported from Japan and Southern Europe, which are usually confined to patients infected with HCV types 1b, 2a and 2b, and to those from the USA where types 1a and 1b predominate. In this study we were able to investigate response to treatment in a large cohort of patients infected with genotype 4 HCV, for which there is currently little information concerning its propensity to cause disease and the extent to which it responds to antiviral treatment.

Table 2 Biochemical responses to IFN-α

Response	*n*	%
Complete responders*	28	30.4
Sustained	18	19.5
Relapse	10	10.8
Non-responders	64	69.6

* Patients showing normalization of ALT levels during treatment, subdivided into those who maintained normal ALT levels in the 6-month post-treatment period, and those who relapsed (see *Materials and methods*).

Table 3 Liver biopsy appearance before and after IFN-α treatment

		Histological improvement	
Biopsy appearance	*n*	*n*	%
CLH*	7	6	86
CPH†	15	12	80
CAH‡	15	6	40
CAH + cirrhosis	14	2	14

* CLH, chronic lobular hepatitis.
† CPH, chronic persistent hepatitis.
‡ CAH, chronic active hepatitis.

In general, an extremely low rate of response was observed. Combining biochemical and virological markers of response, only four of the 92 patients who completed treatment showed sustained normalization of ALT levels and clearance of viraemia 6 months after the cessation of treatment. Furthermore, a high frequency of patients (70%) showed no normalization of ALT even during the treatment period.

The rate of sustained normalization of ALT in this study group was low (20%), and within the range found upon treatment of HCV genotype 1 (9–40%) [9,12,16–20]. This contrasts with rates of 45–91% for genotype 2 [17–19] and 55% for genotype 3 [12]. Similarly, the extremely low rate of sustained virus clearance for type 4 in this study (4%) was similar to that observed for type 1 infected patients (6%) and lower than those infected with type 3 (33%) [20]. Although most of the patients presented with subclinical infection, a high frequency of active liver disease was found upon biopsy. Approximately 25% of the group had CAH and 45% had cirrhosis and this high rate of severe disease compared with other cohorts of patients may have independently contributed to the poor response to treatment [17,18,21–23].

This first large-scale investigation of responses to treatment of Egyptian patients provides no grounds for optimism that conventional IFN-α therapy (3 MU t.i.w.), for infections with HCV genotype 4, will be of benefit for the majority of those treated. The poor rate of response suggests a need for higher dose IFN-α treatment, or IFN-α treatment combined with other antivirals such as ribavirin. This combination (3 MU t.i.w. IFN-α, 1.0–1.2 g ribavirin for 6 months) achieved a sustained virological response (PCR-negative after 6 months from cessation of treatment) in 5/10 patients who had previously failed to respond to interferon monotherapy [24], and may be of value in the study group described here.

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